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Phylogeography and phylogeny of the morphologically highly-variable genus *Acanthonyx* Latreille, 1828 (Crustacea, Decapoda, Epialtidae) in the North-east Atlantic and Mediterranean region

Ana Tavares

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Ana Isabel de Magalhães Tavares

Dissertação de Mestrado apresentada à
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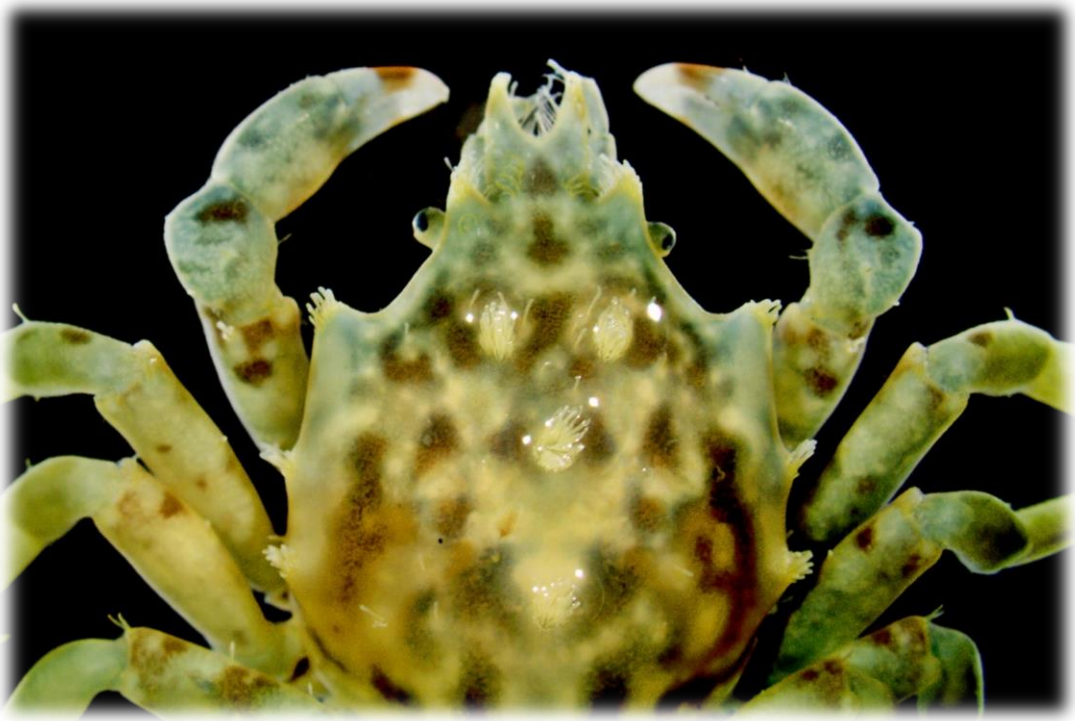

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Mestrado em Recursos Biológicos Aquáticos

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Orientador

António Múrias dos Santos, Professor Auxiliar, Faculdade de Ciências da Universidade do Porto, Centro de Investigação em Biodiversidade e Recursos Genéticos

Coorientador

Pilar Cabezas Rodríguez, Investigadora Post-Doc, Centro de Investigação em Biodiversidade e Recursos Genéticos

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

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Abstract

The superfamily Majoidea Samouelle, 1819 is one of the most diversified groups with about 800 species, commonly known as spider crabs. Although its monophyly is consensual, the internal placement of some families and genera is still far from being resolved. Within Majoidea, the family Epialtidae MacLeay, 1838, is the most species rich. However, for such a diverse group, it is surprising that very few molecular studies have addressed the phylogenetic relationships below the genus level. One such genus, *Acanthonyx* Latreille, 1828 (Majoidea, Epialtidae), currently comprises between 17 or 18 species, depending on competing taxonomic views, and has a world-wide distribution. Morphologically, most species look superficially alike and therefore similar taxonomic concepts have been described under different names. As a consequence, there is today a considerable list of synonyms which further complicates the taxonomy of the genus *Acanthonyx*.

In the Mediterranean and NE Atlantic regions the only species known to occur is *A. lunulatus* (Risso, 1816). Morphological and, to a lesser extent, genetic evidence suggest that this taxonomic concept deserves a much deeper analysis. For one, its southern range limit is thought to be located at Namibia. Such widespread distribution is only compatible with a high capacity for dispersion which, like many decapods, is attained by passive larval dispersal. But larval duration in *A. lunulatus*, although not fully known, is not particularly large (around 15-25 days) when compared to other decapod species. Hence, *A. lunulatus* can potentially display considerable patterns of genetic differentiation. Additionally, a morphologically distinct species - *A. brevifrons* Milne Edwards, 1869 - has been described for the Cape Verde and the Azores, and is either considered a synonym of *A. lunulatus* or as a good species. These standing taxonomical issues and the lack of molecular information prompted for a more thorough study of the phylogeographic patterns of *A. lunulatus*.

An initial phylogeographic analysis including populations of the NE Atlantic, the Mediterranean, and the Macaronesia, using the mitochondrial gene cytochrome c oxidase subunit I (COI), revealed that *A. lunulatus sensu lato* is a complex of three distinct linages: one corresponding to *A. brevifrons*, another to *A. lunulatus sensu stricto*, and a third to a yet undescribed group (named *Acanthonyx* sp.). A phylogenetic analysis of this "species complex" was then made by complementing the mitochondrial data with a nuclear gene (28S ribosomal RNA), and supported the observations made previously. Specifically, *A. brevifrons* deserves the status of a species and can also be distinguished

from *A. lunulatus* by morphological characteristics. So far, this species occurs only in Cape Verde and the Azores, the latter being probably a recent colonization. The degree of genetic divergence of COI between *Acanthonyx* sp. and *A. lunulatus* is below average levels for Decapoda species. Yet, no shared haplotypes have been detected between them. The differences found in the nuclear gene (indels), together with their sympatric occurrence, prompt for a more detailed analysis of this group as no clear morphological distinction has been found. Overall, the results show that significant genetic differentiation between specimens with similar – although highly variable – morphology occurs in the Epialtidae, thus reinforcing the importance of combining morphological and genetic tools to fully resolve the taxonomy of these decapods.

Keywords

Phylogeography, phylogeny, Decapoda, *Acanthonyx*, NE Atlantic, Mediterranean, cryptic species.

Resumo

A superfamília Majoidea Samouelle, 1819 é um dos grupos mais diversificados. Possui cerca de 800 espécies e é vulgarmente conhecido como caranguejos aranha. Embora a monofilia é consensual, a posição interna de algumas famílias e géneros ainda está longe de ser resolvida. Dentro dos Majoidea, a família Epialtidae MacLeay, 1838, é a que possui um maior número de espécies. No entanto, para um grupo tão diverso, é surpreendente a escassez de estudos moleculares que abordem as relações filogenéticas abaixo do nível de género. O género *Acanthonyx* Latreille, 1828 (Majoidea, Epialtidae) que, dependendo dos critérios taxonómicos usados, atualmente tem entre 17 ou 18 espécies, possui uma distribuição cosmopolita. Morfologicamente, a maioria das espécies são superficialmente semelhantes e, portanto, conceitos taxonómicos análogos têm sido descritos sob diferentes nomes. Como consequência, existe hoje uma considerável lista de sinónimos que complica ainda mais a taxonomia do género *Acanthonyx*.

No nordeste Atlântico e Mediterrâneo, *A. lunulatus* (Risso, 1816) é a única espécie descrita para estas regiões. A sua morfologia e a pouca evidência genética sugerem que este conceito taxonómico merece um estudo mais aprofundado. Pensa-se que o limite sul de distribuição é localizado na Namíbia, sendo esta ampla distribuição apenas compatível com uma elevada capacidade de dispersão que, tal como muitos decápodes, é atingida através de dispersão larvar passiva. Apesar da duração larvar de *A. lunulatus* não ser totalmente conhecida sabe-se que não é particularmente longa (cerca de 15-25 dias), quando comparada a outras espécies de decápodes. Assim, esta espécie pode potencialmente ter padrões consideráveis de diferenciação genética. Adicionalmente, a espécie *A. brevifrons* A. Milne Edwards, 1869, descrita para Cabo Verde e Açores é morfologicamente distinta de *A. lunulatus*, mas tem vido a ser considerada tanto como um sinónimo desta, tanto como uma boa espécie. Assim, todos estes problemas taxonómicos e a falta de informação molecular mostram a necessidade de se aplicar um estudo mais aprofundado dos padrões filogeográficos da espécie *A. lunulatus*.

Numa análise filogeográfica inicial, que incluiu populações do Nordeste Atlântico, Mediterrâneo e Macaronésia, utilizou-se o gene mitocondrial citocromo c oxidase subunidade I (COI), tendo revelado que a espécie *A. lunulatus sensu lato* é um complexo de três linhagens distintas: uma que corresponde à espécie *A. brevifrons*, outra a *A. lunulatus sensu stricto,* e um terceiro grupo ainda não descrito (nomeado como *Acanthonyx* sp.). A análise filogenética deste "complexo de espécies" foi realizada,

complementando os dados mitocondriais com dados de um gene nuclear (28S RNA ribossomal), que apoiou as observações feitas previamente. Nomeadamente, no caso da espécie *A. brevifrons* que se conseguiu distinguir através de características morfológicas de *A. lunulatus* e merece o estatuto de espécie. Até agora, esta espécie existe apenas em Cabo Verde e dos Açores, sendo este arquipélago provavelmente uma colonização recente. O grau de divergência genética para a COI entre *Acanthonyx* sp. e *A. lunulatus* está abaixo dos níveis médios para outras espécies de decápodes. No entanto, não foi detetada qualquer partilha de haplótipos entre estas duas espécies. As diferenças foram no entanto encontradas no gene nuclear (indels) que, juntamente com a ocorrência em simpátrica, evidenciam a necessidade de uma análise mais detalhada de *Acanthonyx* sp. já que nenhuma distinção morfológica clara foi encontrada entre esta e *A. lunulatus*. Em suma, os resultados demostram que uma diferenciação genética significativa entre indivíduos com morfologia semelhante, embora altamente variável, existem no grupo Epialtidae, reforçando assim a importância de combinar ferramentas morfológicas e genéticas para resolver totalmente a taxonomia destes decápodes.

Palavras-chave

Filogeografia, filogenia, Decapoda, *Acanthonyx*, Nordeste Atlântico e Mar Mediterrâneo; espécies crípticas.

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List of Abbreviations

AIC – Akaike Information Criterion

AOF – Almeria–Oran Front

BI – Bayesian inference

BP – Bootstrap support

BSA – Bovine Serum Albumin

COI – Cytochrome c oxidase subunit I

DNA – Deoxyribonucleic Acid

dNTP – Deoxynucleotide

EF1α – Elongation Factor 1 α gene

H3 – Histone H3 GENE

ICZN – International Code of Zoological Nomenclature

K2P – Kimura 2-parameter

MCMC – Markov chain Monte Carlo

MgCl2 – Magnesium chloride

ML – Maximum likelihood

mtDNA – Mitochondrial DNA

MSC – Messinian salinity crisis

Mya – Million years ago

NCBI – National Center for Biotechnology Information

nDNA – Nuclear DNA

NE – Northeast

PCR – Polymerase Chain Reaction

PP – Bayesian posterior probability

RNA – Ribonucleic Acid

TRCA – Time to the most recent common ancestor

1. Introduction

Among true crabs (Decapoda, Brachyura), the superfamily Majoidea Samouelle, 1819 is one of the most diversified and is thought to contain more than 800 species of the commonly known spider crabs (Rice, 1988). These are distributed in six families (*sensu* Ng *et al.,* 2008), which is the adopted taxonomy in the current work)- Epialtidae MacLeay, 1838; Hymenosomatidae, MacLeay, 1838; Inachidae MacLeay, 1838; Inachoididae Dana, 1851; Majidae Samouelle, 1819 and Oregoniidae Garth, 1958. Historically, it has been a problematic group, undermined by several ambiguities at the level of family/subfamily definitions and their hierarchical placement (see Ng *et al.,* 2008 for a brief revision). Although the monophyly of the Majoidea is consensual (Hultgren and Stachowicz, 2008), the incorporation of molecular and larval morphology data into phylogenetic reconstructions showed that the internal placement of some families and genera is still far from being resolved (Hultgren and Stachowicz, 2008; Hultgren *et al.,* 2009; Mahon and Neigel, 2008; Marques and Pohle, 2003).

Within Majoidea, the family Epialtidae MacLeay, 1838, is the most species rich, with around 380 species distributed by four sub-families (Epialtinae MacLeay, 1838; Tychiinae Dana, 1851; Pisinae Dana, 1851 and Pliosomatinae Stevcic, 1994) and probably one of the most morphologically heterogeneous (Colavite *et al.,* 2014). Consequently, it suffers from the exact same issues of its parent taxon. Given the popularity of this group, more than half of its species were described before the 1900s and placed into different genera, contributing substantially to the current intricate list of synonymies (Ng *et al.,* 2008). Adding to this complexity, new species, and even new genera, are still being described (e.g. Forges and Ng, 2009; Ng and De Forges, 2013; Tavares and Santana, 2011). For such a diverse group, it is surprising that very few molecular studies have addressed the phylogenetic relationships below the genus level (e.g. Gomes, 2013). This also applies to the Majoidea as a whole, where only *Mithrax* Desmarest, 1823, *Mithraculus* White, 1847, and *Maja* Lamarck, 1801, were subject of phylogenetic analysis (Baeza *et al.,* 2009; Sotelo *et al.,* 2009; Windsor and Felder, 2009). The NCBI Taxonomy Browser (Benson *et al.,* 2009) lists 28 epialtid species (plus two environmental samples) for which nucleotide data is available, whereas the BOLD System V3 (Ratnasingham and Hebert, 2007) comprises 46 barcodes. Yet, most of these data result from the supra-generic analysis mentioned before (see Hultgren *et al.,* 2009) and not from research targeted on specific genera.

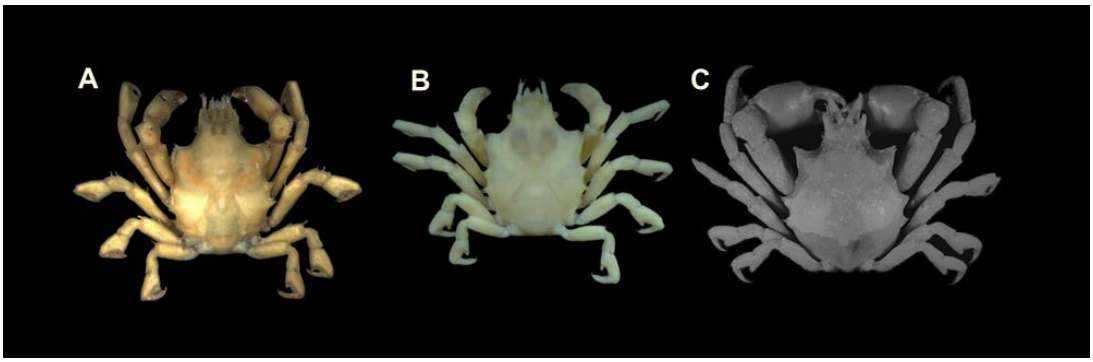

Figure 1 - Three *Acanthonyx* species **-** A: *Acanthonyx petiverii* H. Milne Edwards, 1834; B: *Acanthonyx dissimulatus* Coelho, 1993; C: *Acanthonyx formosa* Wu, Yu & Ng, 1999. Image A and B adapted from Gomes (2013) and C from Yu *et al.* (1999).

1.1 The genus *Acanthonyx* Latreille, 1828

The genus *Acanthonyx* Latreille, 1828 currently comprises between 17 and 18 species (see Emparanza *et al.,* 2007; Ng *et al.,* 2008) listed in Table 1. It is a cosmopolitan genus, with species described from coastal habitats of the American and African continents, the Macaronesia, the Mediterranean Sea, and several regions of the Indo-Pacific (Chace, 1966; Emparanza *et al.,* 2007; Griffin and Tranter, 1986; Manning and Holthuis, 1981; Rathbun, 1925).

The morphology of most species is superficially similar (see Figure 1) and the most important diagnostic characters are the shape of the carapace, its ornamentation, and the shape of rostral sinus. Because these are not discrete traits, the same taxonomic concepts have been given different names (species described independently) and synonyms are not hard to find (see Table 1). Yet, there is no comprehensive revision of this genus, and identification keys cover only non-overlapping, albeit large, regions such as the eastern Atlantic (Manning and Holthuis, 1981), and the Indo-West pacific (Griffin and Tranter, 1986). A global treatment of American species is still absent, despite some recent taxonomic and nomenclatural rearrangements (Emparanza *et al.,* 2007). For example, some authors suggest that *A. dissimulatus*, *A. scutiformis* and *A. simplex*, should be synonymized with *A. petiverii* (Emparanza *et al.,* 2007; Gomes, 2013). Another unresolved case is *A. brevifrons* Milne-Edwards, 1869, which was described from Cape Verde islands and, according to its author, also occurred in Azores. Given its superficial similarity with *A. lunulatus,* Alvarez (1968) deemed *A. brevifrons* as a synonym of the latter. This taxonomic view was supported by several authors, either in the late 1800s (Miers, 1886) or more recently (Emparanza *et al.,* 2007; Ng *et al.,* 2008)

Table 1 - Extant species of the genus *Acanthonyx* and known synonyms according to Ng *et al.* (2008).

Current name	Synonyms
Acanthonyx consobrinus A. Milne-Edwards, 1862	
Acanthonyx dentatus H. Milne Edwards, 1834	*Dehaanius acanthopus* MacLeay, 1838
Acanthonyx depressifrons Manning & Holthuis, 1981	
Acanthonyx dissimulatus Coelho, 1993	
Acanthonyx elongatus Miers, 1877	
Acanthonyx euryseroche Griffin & Tranter, 1986	
Acanthonyx formosa Wu, Yu & Ng, 1999	
Acanthonyx inglei Tirmizi & Kazmi, 1988	
Acanthonyx limbatus A. Milne-Edwards, 1862	
Acanthonyx lunulatus (Risso, 1816)	*Inachus levigatus* Rafinesque, 1814 *Maia glabra* Latreille, 1836 *Acanthonyx viridis* Costa, 1838 *Gonosoma viridis* Costa, 1844 *Acanthonyx brevifrons* A. Milne-Edwards, 1869
Acanthonyx minor Manning & Holthuis, 1981	
Acanthonyx nodulosa (Dana, 1852)	
Acanthonyx petiverii H. Milne Edwards, 1834	*Acanthonyx simplex* Dana, 1852 *Acanthonyx emarginatus* H. Milne Edwards & Lucas, 1843 *Acanthonyx debilis* Dana, 1851 *Acanthonyx concamerata* Kinahan, 1857
Acanthonyx quadridentatus Krauss, 1843	
Acanthonyx sanctaehelenae Chace, 1966	
Acanthonyx scutellatus MacLeay, 1838	*Acanthonyx macleaii* Krauss, 1843
Acanthonyx scutiformis (Dana, 1851)	
Acanthonyx undulatus Barnard, 1947	

but according to others the two species should be considered as distinct (Manning and Holthuis, 1981). The lack of phylogenetic studies, combined with poor diagnosis or descriptions and the morphologic similarities between some species led to a great deal of taxonomic confusion and more research should be done to clarify the existing doubts about the genus *Acanthonyx*.

1.2 *Acanthonyx lunulatus* (Risso, 1816)

Acanthonyx lunulatus was described by Risso (1816) as *Maia lunulatus* from Nice (France), but was later moved into the genus *Acanthonyx* by Latreille (1828). In this early stage of crustacean taxonomy, several species were independently described under different names (see Table 1). *Inachus levigatus* Rafinesque, 1814, is a special case, because the name has priority over *lunulatus* from Risso (1816), but given that the latter has become firmly established in carcinological literature, the former has been suppressed (ICZN, 1959).

A. lunulatus, like other species of the genus, inhabits shallow-waters on rocky substrates, usually attached to algae (Alvarez, 1968; Manning and Holthuis, 1981). It is currently the only species of the genus *Acanthonyx* known to occur in the Mediterranean and NE Atlantic regions, where it extends from southern Portugal to Namibia, reaching the Kunene River (Manning and Holthuis, 1981). Such widespread distribution suggests a high capacity for dispersion. For many coastal marine species, larvae rather than adults are the true responsible for dispersion. If they are minute and have small to none swimming ability, dispersion is passive and driven solely by currents (Cowen and Sponaugle, 2009). Population connectivity will then depend only on larval duration (García-Merchán *et al.,* 2012). Like most brachyurans, dispersion in Epialtidae is probably driven by larval stages, although adult rafting in seaweed cannot be excluded, since it was reported for a few species, such as *Hyas* Leach, 1814, and *Macropodia* Leach, 1814 (see Thiel and Gutow, 2005 for a review).

As most benthic marine invertebrate species, which have complex life cycles, the development of *A. lunulatus* includes two zoea and one megalopa, thus following the typical pattern in the Majoidea (Guerao and Abelló, 1996; Kornienko and Korn, 2007). However, larval duration for *A. lunulatus* is not completely known: the two zoea stages can endure a maximum of 15 days (Guerao and Abelló, 1996), but this might be longer if the megalopa is also involved in dispersal. Larvae of small Majoidea typically vary between 15-25 days (e.g. Colavite *et al.,* 2014; Kornienko and Korn, 2007; Santana *et al.,* 2004a; 2004b; Santana and Marques, 2009), hinting at low levels of genetic structure.

However, other Atlantic-Mediterranean invertebrate species with comparable larval duration have revealed considerable patterns of genetic differentiation (Sá-Pinto *et al.,* 2008; Zulliger *et al.,* 2009), and even cryptic species (Sá-Pinto *et al.,* 2005). Thus, *A. lunulatus* can potentially display similar differentiation patterns, but phylogeographic studies of epialtids are non-existent, and this particular species has mostly been used as outgroup in phylogenetic analysis of other epialtid genus (Windsor and Felder, 2014) or higher taxa (Silva *et al.,* 2011). Such conflicting ideas, standing taxonomical issues and a gap of molecular information, prompt for a more thorough study of the phylogeographic patterns of *A. lunulatus*.

1.3 The Mediterranean Sea

The Mediterranean Sea is a mid-latitude semi-enclosed marine basin, an almost isolated oceanic system, surrounded by large continental masses. This basin has a connection to the Atlantic Ocean through the Strait of Gibraltar and through the Dardanelles to the Sea of Marmara and the Black Sea in the northeast. In the southeast, the man-made Suez Canal (opened in 1869) links the Mediterranean to the Red Sea and the Indian Ocean (Coll *et al*., 2010). The Mediterranean is formed by two principal sub-

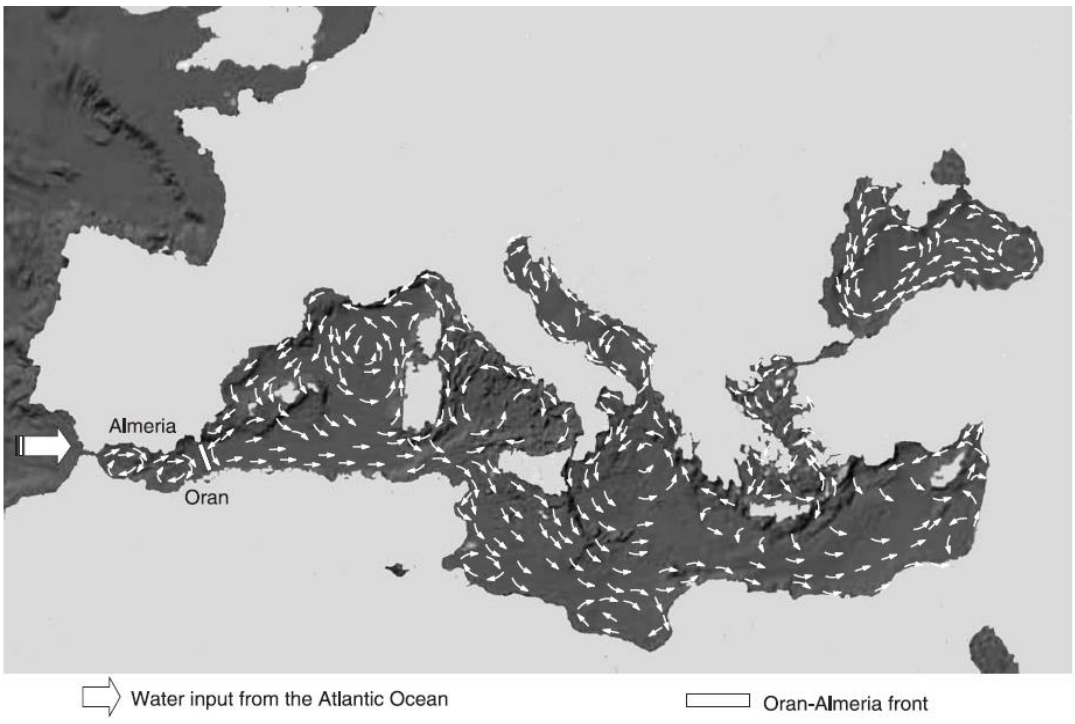

Figure 2 - Representation of the main currents illustrating water circulation in the Mediterranean and Black Sea (Patarnello *et al.,* 2007)

basins - the Western and the Eastern Mediterranean - connected by the Strait of Sicily (Astraldi *et al*., 1999). Both enclose several regional seas that are also separated by straits and channels. It has an average depth of approximately 3400m in the western basin and about 4200m in the eastern basin (Millero, 2013).

The Mediterranean circulation (Figure 2) is forced by water exchange through the various straits, wind stress, and buoyancy flux at the surface due to fresh water and heat fluxes (Robinson *et al.,* 2001). Since this Sea is situated in the Northern of the desert climatic belt, there is a significant net loss of water from this basin and, particularly, in the eastern basin, there has a large excess of evaporation over precipitation. These results in an anti-estuarine circulation and locally deep convection characterize the Mediterranean Sea (Black and Shimmield, 2009). The patterns of water circulation in the Western Mediterranean are characterized by the inflow of surface Atlantic water and outflow of deeper Mediterranean water (Millot, 2005). Less dense Atlantic water enters at the surface through the Strait of Gibraltar with an average salinity of 36.15 ppt (Millot, 1999). Salinity, due to evaporation, tends to increase from the west to east generating a west-east gradient with a maximum in the eastern Mediterranean (Coll *et al.,* 2010). The inflowing Atlantic water forms in the Alboran Sea a permanent dynamic oceanographic zone allowing the connection to the main jet of incoming Atlantic water and the Mediterranean Sea.

1.3.1 Historical events: geological and climatic changes

Genetic variability and population genetic structure of species are shaped by both past and present processes. Consequently, a deep understanding of the geological, climatic and oceanographic processes are needed to interpret current patterns of genetic diversity. The Mediterranean Sea is one of the most important hot-spots of marine biodiversity and represents of 4% to 18% of world's marine biodiversity (Coll *et al.,* 2010; Patarnello *et al.,* 2007). A possible explanation for this diversity could be attributed to the historical interest of naturalists, but it may also have origin in the troubled geological history of the Mediterranean region and its drastic environmental changes (Bianchi and Morri, 2000; Coll *et al.,* 2010). Two major historical events are thought to be the causes of its current diversity: the Messinian salinity crisis (MSC) and the Quaternary glaciations (Patarnello *et al.,* 2007).

In the late Miocene, the connection between the Atlantic and Mediterranean was interrupted due to complex tectonic, volcanic and glacio-eustatic events (Duggen *et al.,* 2003), known as the Messinian Salinity Crisis (MSC). The MSC happened between 5.59

and 5.33 Mya ago (Krijgsman *et al.,* 1999). This event led to the almost desiccation of the Mediterranean, climate and sea level changes and, consequently to the precipitation of high levels of salt. This, in turn, probably drove to extinction almost all marine species, except possibly those that remained in areas that received water from rivers (e.g. Carballo *et al.,* 1997; Sotelo *et al.,* 2009). The MSC ended with the opening of the Strait of Gibraltar and, because of tectonic uplifting, faulting and sea level changes, the Mediterranean was flooded with oceanic water, at the beginning of the Pliocene (Carballo *et al.,* 1997; Patarnello *et al.,* 2007).

Pleistocene glaciations were the other historical process that modified the diversity of the Mediterranean Sea. The alternation of the ice ages with the warm inter - glacials during the Quaternary probably forced the retraction of marine species into warmer regions, which were probably refugia for many temperate species. Some of such locations were located in the Mediterranean and southern regions of the Northeast Atlantic, like North African coasts and the Macaronesia (Almada *et al.,* 2005; Xavier *et al.,* 2011).

1.3.2 Population structure across the Atlantic - Mediterranean transition zone

The rich geological history of the Mediterranean, together with the complex past and contemporary climate and seawater currents, clearly shaped the diversity of species in this water body (Patarnello *et al.,* 2007). Additionally, biological (e.g. life history traits, larval behaviour) and ecological (e.g. food availability, species interactions) processes also contributed to shape the patterns of genetic diversity within species, in many cases leaving a clear signature on the connectivity and genetic structure of populations (see Grantham *et al.,* 2003; Palumbi, 2004; White *et al.,* 2010). For example, transition zones between two seas or oceans are known to impact the phylogeographic structure of many species. Many studies have been focused in these transition zones with the intent to understand how they shape the structure of populations and gene flow between them (Avise *et al.,* 1987; Riginos *et al.,* 2011).

Within the Mediterranean Sea, there are several barriers to dispersal and connectivity and one of the most well-known is found in the Atlantic – Mediterranean transition zone, at the east of the Alboran Sea, called the Almeria–Oran Front (AOF). The AOF is formed due to the convergence of the low-salinity Atlantic waters and the saltier Mediterranean waters. There is evidence that this front acts as a barrier to gene flow for many species, either passive or active dispersers (see Patarnello *et al.,* 2007 for

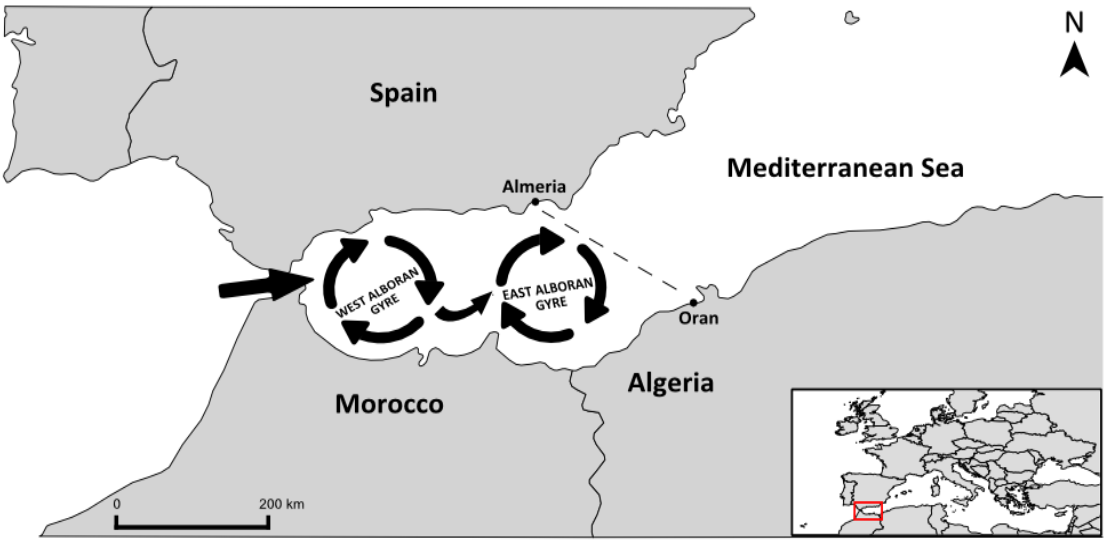

Figure 3 - Schematic representation of the anticyclonic gyres of the Alboran Sea. Dashed line represents the Almeria-Oran Front.

a review), and it is thought to be the major genetic break between the Atlantic and the Mediterranean, even when compared to the more obvious Strait of Gibraltar. Nonetheless, many species are also not affected by the AOF (Bargelloni *et al.,* 2005; García-Merchán *et al.,* 2012).

The AOF and the Strait of Gibraltar delimit the Alboran basin, which acts as a transition zone between the Atlantic and the Mediterranean. It is characterized by two semi-permanent gyres (Figure 3), the Western and the eastern Alboran gyres (Tintore *et al.,* 1988), and consequently displays complex patterns of water circulation (see Skliris and Beckers, 2009 for a review). Genetic differentiation across this transition zone has been shown for many marine taxa, including crustaceans (Palero *et al.,* 2008), echinoderms (Duran *et al.,* 2004), isopods (Xavier *et al.,* 2011) and molluscs (Pérez-Losada *et al.,* 2002).

1.4 Cryptic species and diversity

Morphology (the study of the physical traits of an organism) has been used as the main tool for taxonomists and biologists to describe and identify species (Mayr, 1942). However, and because speciation is not always followed by morphological changes, biodiversity is probably higher than the known current number of species (Bickford *et al.,* 2007). Molecular tools have proved very useful in the identification of species and in uncovering high levels of genetic diversity. The marine environment is a very complex

habitat and molecular advances (e.g. genetic barcodes) provide other means to study marine biodiversity and allow the identification/recognition of new species, particularly those that are cryptic, *i.e.*, which are morphological indistinguishable but genetically distinct and reproductively isolated (Bickford *et al.,* 2007). The concept of cryptic species is not new (Sáez and Lozano, 2005) and in the last decades, evidence showed that the number of cryptic species is increasing, mostly because of the successful application of molecular tools (Bickford *et al.,* 2007). This scenario is similar in the marine environments, with reports of marine cryptic species growing, namely in the case of marine invertebrates (e.g. Blanquer and Uriz, 2007; Derycke *et al*., 2010; Knowlton, 2000). A considerable number of cryptic species from the Decapoda group have been found (e.g. Bilgin *et al*., 2014; Silva *et al.,* 2011; Tourinho *et al.,* 2012; Tsoi *et al.,* 2014). Due to the extensive variability of certain morphological characters, many of them used in species diagnosis, it would be expected to found new cryptic species among widely-distributed decapods (Silva *et al.,* 2011). Finally, and because the taxonomy of the genus *Acanthonyx* is not yet completely resolved, and a phylogeny of the genus is lacking, the hypothesis of the existence of cryptic species in this genus cannot be rejected without further evidence.

1.5 Molecular tools and genetic markers

Beyond morphology, the actual evolutionary research is usually based on DNA sequence data. The genetic information is transmitted from generation to generation through the DNA of the previous descendants (Allan and Max, 2010). The whole diversity observed today is a consequence of this transmission with a gradual accumulation of successive modifications (e.g. inversions, duplications, and translocations), which allow organisms to change (Lemey, 2009). Evolutionary forces such as selection, genetic drift and gene flow, led to modifications visible at several taxonomic levels, from species to higher groups (Avise, 2012; Park and Moran, 1994). Molecular techniques take advantage of this and are applied in several areas allowing a better understanding of the biodiversity observed today.

In animals, mitochondrial DNA (mtDNA) is a circular molecule. It has a small genome size, normally with 37 genes and around 17,000 nucleotide base pairs with no introns or large non-coding regions (Boore, 1999) and it can be easily obtained given that it occurs in high copy numbers in every cell. It is usually inherited from the female parent and so, each copy is identical, having a non-recombining mode of inheritance. It

evolves rapidly in animal populations and has extensive intraspecific polymorphism (e.g. Avise, 2000; Féral, 2002). Hence, mtDNA is appropriate to infer evolutionary relationships and it has significantly contributed to the establishment of phylogeography (Avise *et al.,* 1987; Avise, 2000). Usually, mtDNA has a lower effective population size (due to the strict maternal inheritance), offering an advantage over nuclear DNA (nDNA) on phylogenetic studies, due to a more rapid fixation of new haplotypes. Furthermore, mtDNA mutation rates are typically higher than in nuclear DNA and so, it is not expected to show incomplete lineage sorting (see Ballard and Whitlock, 2004 for a review). However, genetic saturation occurs more rapidly on fast evolving molecules with higher mutation events and consequently, decreases its utility in phylogenetic inference.

The mitochondrial gene cytochrome c oxidase subunit I (COI), one specific region of mtDNA, as proved particularly informative at lower taxonomic levels (Allan and Max, 2010). In recent years, this gene, has been used on barcoding studies due to the facility on the application of universal primers and, more than any other gene, COI seems to have a higher signal reach on phylogenetic studies (Hebert *et al.,* 2003). In studies of crustacean species, the COI gene offers a good resolution to discriminate different taxonomic levels (Costa *et al.,* 2007). Within the Crustacea, decapods are the most recognizable and dominant group and the COI has been used successfully on the assessment of decapod biodiversity (e.g. Puillandre *et al.,* 2011; Silva *et al.,* 2011).

More recently, some researchers concluded that, even though mtDNA may be extremely useful in phylogenetic studies due to its characteristics, its usage alone is not sufficient to resolve complex questions about the history of populations (Godinho *et al.,* 2008). The application of mtDNA alone (even if using multiple genes) provides only knowledge of the history of a single locus and may reflect only a slight part of a species evolutionary history, leading to serious biases or mistakes (Zhang and Hewitt, 2003). Due to the above mentioned drawbacks, it has been common to find phylogenetic studies based on multilocus datasets, frequently with mitochondrial and nuclear genes, thus allowing more robust inferences in studies of evolutionary relationships. Following this, in this study two different genes were used on phylogenetic analysis.

The nuclear DNA differs from mtDNA in the type of inheritance (biparental), the possibility of recombination, and by having a higher effective population size and a lower ploydy and mutation rate (Ballard and Whitlock, 2004). Nuclear DNA can bring some difficulties when used on phylogeographic studies due to several characteristics such as recombination, heterozygosity, rate variation and also amplification and sequencing (Zhang and Hewitt, 2003). In phylogeographic and phylogenetic analyses of crustacean species, several nuclear genes have been used. On phylogeographic studies it is

common to use the Elongation Factor 1 α gene (EF1α) (e.g. Xavier *et al.,* 2012), but for phylogenetic analysis, genes such as Histone H3 GENE (H3) and the 28S ribosomal DNA are preferred (e.g. Hultgren and Stachowicz, 2008; Xavier *et al.,* 2011).

1.6 Objectives

The initial aim of this work was to determine the phylogeographic patterns of genetic variation of *Acanthonyx lunulatus* in the Mediterranean and the Northeast Atlantic, including the Macaronesia. However, in the early stages of this study, evidence of significant morphological differentiation between some *A. lunulatus* "lineages", specifically between Cape Verde + Azores and the Mediterranean, prompted for a more complex analysis, namely by inferring the phylogeny of the NE Atlantic "lineage" of this genus. So, in this thesis I investigate the levels of genetic differentiation of a widely distributed species using a mitochondrial gene (cytochrome c oxidase subunit I) and a nuclear gene (28S ribosomal RNA). Specifically, I aim to: (i) analyse the phylogeographic patterns of *A. lunulatus* (*sensu stricto*, that is, excluding the specimens from Cape Verde and Azores) in the NE Atlantic-Mediterranean region; (ii) analyse the phylogeny of the NE Atlantic *Acanthonyx* lineages; and (iii) try to date the origin of these lineages and associate them with known geological events, since substitution rates for the cytochrome c oxidase subunit I were available for other crab species.

2. Methodologies

2.1 Sampling

A total of 197 *Acanthonyx* individuals were collected in the course of several field trips along the Macaronesian archipelagos of the Azores, Selvagens and Canaries as well as in several localities in de Atlantic-Mediterranean region (Figure 4; Table 2). These expeditions were conducted between 2007 and 2014, and included a total of 36 sampling sites. Individuals were collected among brown and red algae. Algae were collected on the lower intertidal during spring tides and were immediately washed with fresh water to extract all specimens. These were sorted in the field and stored in 96% ethanol.

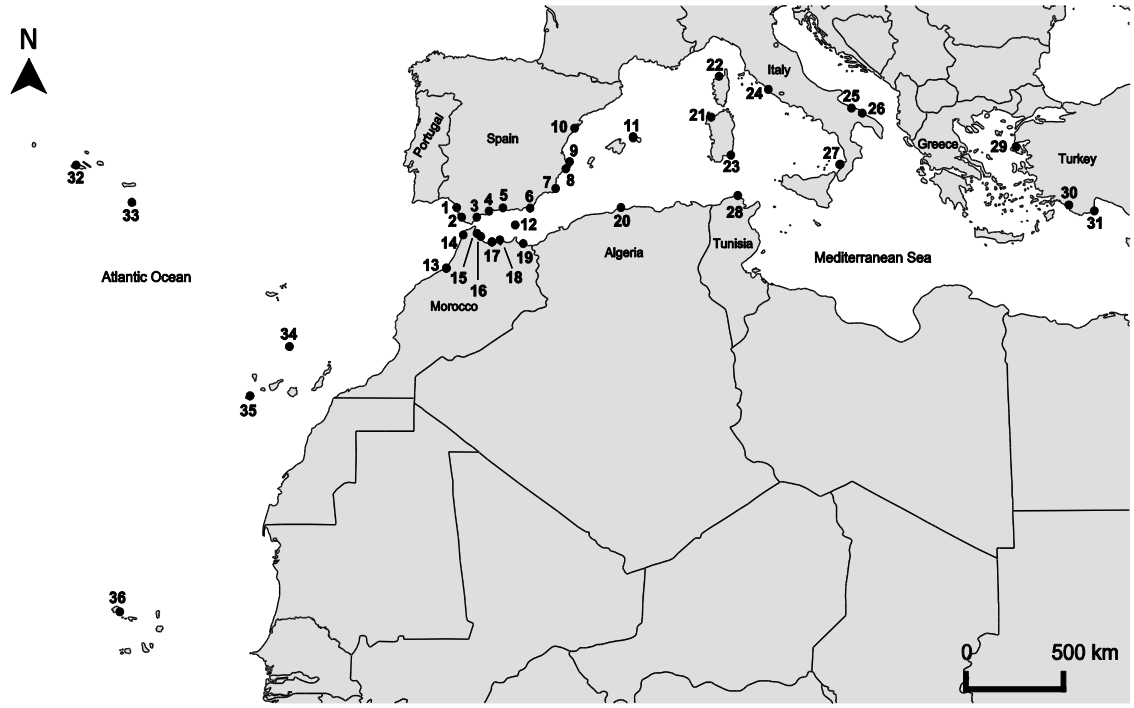

Figure 4 - Map of sampling localities included in the present work. Localities: 1. Chipiona; 2. Conil; 3. Torreguadiaro; 4. Benalmadena; 5. Herradura; 6. Cabo da Gata; 7. Cabo de Palos; 8. Cala del Tío Ximo; 9. Dénia; 10. Peniscola; 11. Cala Blanca; 12. Alboran Island; 13. Temara and Le Falouque; 14. Asilah; 15. Azla; 16. Zaouia; 17. Dos Torres; 18. Al Hoceima; 19. Raselma Nador; 20. Algiers; 21. Porto Torres; 22. L'Île-Rousse; 23. Simius; 24. Santa Marinella; 25. Giovinazzo; 26. Monopoli; 27. Formiggi beach; 28. Bizerte; 29. Molivos; 30. Fethiye; 31. Çirali Limani; 32. Monte da Guia; 33. Santa Maria; 34. Selvagens; 35. El Hierro; 36. Mindelo.

Table 2 - List of individuals included in the phylogenetic analysis, their codes and sampling sites with respective geographical coordinates.

Locality	Country	n	Year	Coordinates
Algiers	Algeria	1	2001	36.749,3.086
Mindelo	Cape Verde	17	2014	16.876,-25.001
L'Île-Rousse	France	2	2010	42.634,8.944
Molivos	Greece	1	2008	39.368,26.172
Formiggi beach	Italy	1	2010	38.664,15.853
Giovinazzo	Italy	5	2010	41.184,16.680
Monopoli	Italy	1	2010	40.958,17.294
Porto Torres	Italy	1	2008	40.836,8.420
Santa Marinella	Italy	1	2010	42.044,11.826
Simius	Italy	5	2008	39.128,9.530
Al Hoceima	Morocco	5	2013	35.251,-3.919
Asilah	Morocco	16	2010	35.475,-6.031
Azla	Morocco	3	2013	35.547,-5.236
Dos Torres	Morocco	1	2012	35.150,-4.367
Le Falouque (Rabat)	Morocco	5	2010	33.920,-6.969
Raselma Nador	Morocco	12	2013	35.078,-2.564
Témara	Morocco	7	2010	33.914,-6.980
Zaouia	Morocco	12	2013	35.403,-5.016
Alboran Island	Spain	4	2014	35.945,-3.034
Benalmadena	Spain	6	2014	36.580,-4.558
Cabo de Gata	Spain	5	2008/2014	36.724,-2.177
Cabo de Palos	Spain	8	2014	37.631,-0.699
Cala Blanca	Spain	2	2010	39.975,3.831
Conil	Spain	4	2014	36.296,-6.133
Cala del Tío Ximo	Spain	22	2014	38.529,-0.105
Chipiona	Spain	1	2007	36.741,-6.436
Dénia	Spain	1	2008	38.848,0.109
El Hierro	Spain	1	2014	27.743,-18.021
Herradura	Spain	9	2014	36.737,-3.756
Peniscola	Spain	3	2008	40.359,0.406
Torreguadiaro	Spain	4	2014	36.300,-5.265
Bizerte	Tunisia	2	2009	37.284,9.877
Çirali Limani	Turkey	3	2010	36.413,30.480
Fethiye	Turkey	1	2010	36.697,29.033
Monte da Guia	Portugal	4	2014	38.519,-28.627
Santa Maria	Portugal	13	2015	37.010,-25.102
Selvagens	Portugal	8	2014	30.145,-15.864

Table 3 - Selected characters for taxonomic revision of the species of *Acanthonyx.*

Morphological characters	
Carapace	Format; number of lateral lobes
Walking legs	Dactyli tubercles number; Periopod shape
Chelipeds	Number of lobes or tubercles; Length
Gonopod	Shape
Abdomen	Number of abdominal somites ; Shape
Rostral sinus	V-shape or U-shape
Rostral teeth	Length

2.2 Taxonomic revision

All specimens of *Acanthonyx* were observed at a binocular microscope. Specimens were firstly compared with the diagnosis of Alvarez (1968) which considers a single species (*A. lunulatus*) for the whole NE Atlantic, including the Macaronesia. Once I realized that the specimens from Cape Verde differed almost consistently in a single trait (the number of lateral lobules in the carapace), I switched to the diagnosis of (Manning and Holthuis, 1981), which distinguish *A. lunulatus* from *A. brevifrons.* The latter species is restricted to Cape Verde and the Azores. In this diagnosis, the most important morphological characteristics used are summarized in Table 3.

2.3 DNA extraction, amplification and sequencing

At the CIBIO laboratory, genomic DNA was extracted from muscle tissue of each crab using the commercial kit Jetquick (Genomed). The tissue was taken from the terminal part of one leg or from one entire leg as these are taxonomically less relevant in species identification. This allowed the preservation of most of the specimens for further morphological inspection if necessary (when individuals were too small, the whole specimen was used). DNA amplification was achieved through PCR for one mitochondrial fragment – cytochrome c oxidase subunit 1 (COI) – and one nuclear fragment – 28S ribosomal RNA. The latter was only amplified in a subset of 28 representative individuals of each major clade (see section “Results”). PCR conditions and primers are described on Table 4.

Table 4 - Gene, sequence, source and PCR conditions (temperature, time and number of cycles).

Gene	Sequence (5'- 3')	Reference	PCR condition
COI	F:TITCIACIAAYCAYAARGAYATTGG R:TAIACYTCIGGRTGICCRAARAAYCA	Geller *et al.,* 2013	94ºC(4m), [40x 94ºC(45s), 45ºC(50s), 72ºC(1m)], 72ºC(10m)
28S	F:GCAGTCTCTCACCGCCTAAGTTAT R:GACTCCTTGGTCCGTGTTTCAAGA	Hultgren and Stachowicz, 2008	94ºC(4m), [40x 94ºC(30s), 65ºC(1m), 72ºC(1.30m)], 72ºC(10m)

Polymerase Chain Reaction (PCR) amplifications were performed with a final 25 µL volume for each sample, according to Invitrogen™, consisting of 3 µl template DNA, -10x buffer $MgCl_2$ free, 3mM $MgCl_2$, 0.2 mM dNTP, 1µM of each primer, 0.1 µg $µl^{-1}$ Bovine Serum Albumin (BSA, Promega), 0.3U Platinum Taq DNA polymerase, and double-distilled water to volume. PCR were run in a Biometra TProfessional thermal cycler. Two controls were added for each run – a positive control, to verify that PCR reaction was effective by using a sample that was previously amplified for the gene in question; and a negative control, which has all reagents except DNA to check for contaminations. To confirm the success of amplification, 3 µL of PCR product from each sample were used in a 2% (w/v) agarose gel with GelRed (DNA fluorescent dye; BioTarget). Gels were run at 300V and the extracted DNA was visualized in a UV transilluminator device (Bio-Rad). Pictures of each gel run were taken and saved. Amplification products were sent to Beckman Coulter Genomics (UK) for purification and Sanger sequencing with the same primers used in amplification. The nuclear gene was sequenced in both directions to ensure identification of heterozygotes.

Sequences obtained were blasted to the NCBI database on GenBank to confirm the species identification. Chromatograms were checked and sequences were aligned for posterior phylogenetic analysis using CodonCode Aligner 4.2.4 (CodonCode, Dedham, MA, USA). Previously published sequences (GenBank) of 28S EU682903 (Hultgren *et al.,* 2009) and COI JQ305885.1 (Silva *et al.,* 2011); KF452903.1 (Windsor and Felder, 2014); EU62854, EU682855 (Hultgren *et al.,* 2009); KC695765, KC695767, KC695771–KC695773, KC695775 (Tamburus and Mantelatto, unpublished data) *o*f *Acanthonyx* were added to the data set.

2.4 Estimates of genetic diversity and population structure

Two measures of mtDNA diversity were estimated for each locality, using ARLEQUIN, version 3.5.1.2 (Excoffier and Lischer, 2010): haplotype diversity (Hd), that indicates the probability that two randomly chosen haplotypes can differ in a population; and nucleotide diversity (π), that shows the percentage mean number of differences between all pairs of haplotypes in a population. Neutrality tests, Tajima's D and Fu's FS, were also estimated. Localities with less than four individuals or that displayed no polymorphism were omitted from the analyses. So, in this analysis, twenty locations - Algiers, L'Île-Rousse, Molivos, Azla, Dos Torres, Cala Blanca, Cap d'Artrutx, Cala de sant Vicenç, Chipiona, Dénia, El Hierro, Peniscola, Porto Torres, Santa Marinella, Formiggi beach, Monopoli, Tropea, Bizerte, Çirali Limani and Fethiye – were excluded (Table 6).

Relationships between mtDNA haplotypes was further investigated by building an haplotype network (see Figure 8) using the statistical parsimony procedure of Templeton *et al*. (1992) implemented in TCS version 3.5.1.2 (Clement *et al.,* 2000), plotted with tcsBU (Santos *et al.*, 2015). Gaps were coded as missing data and a 95% statistical parsimony connection limit was used.

2.5 Phylogenetic analysis

The amino acid translation was examined for all the sequences, to ensure that no gaps or stop codons were present in the alignment. BLAST searches were performed via GenBank online nucleotide database. Given the preliminary evidence of high levels of genetic differentiation between three lineages within *A. lunulatus*, one of which corresponds to a described species (*A. brevifrons*), Kimura 2-parameter (K2P) and raw (p) distances for the mitochondrial gene using MEGA version 6 (Tamura *et al.,* 2013) were computed. The dataset included three common haplotypes for each of the three *A. lunulatus* lineages, plus 10 sequences from GenBank identified as *A. lunulatus* (2), *A. scutiformis* (1), *A. dissimulatus* (1), and *A. petiverii* (7). The latter three species are all from SW Atlantic.

The appropriate models of nucleotide substitution were calculated using JModelTest 2.1.7 (Darriba *et al.,* 2012), and Akaike Information Criterion (AIC) was used to select the appropriate molecular evolution models for the COI and 28S datasets, plus a concatenated dataset (COI + 28S). In this study, I applied two model-based methods of phylogenetic inference - Bayesian inference (BI) and maximum likelihood (ML).

Phylogenetic analyses were performed for the different datasets using MrBayes 3.2.2 (Ronquist *et al.,* 2012) for BI and Garli 2.0.1 (Zwickl, 2006) for ML. Analyses were made using two data partitions (1+2, 3 codon positions) for the mitochondrial gene, to minimize saturation effects of third positions on phylogenetic reconstructions. For Bayesian analysis, two separate runs were conducted for 3×10^7 generations each, and trees and parameters were sampled every 1000 generations with the heating parameter set to 0.25. Majority-rule consensus trees were estimated combining results from duplicated analyses, after discarding the first 7500 samples as burn-in (corresponding to 25% of the total samples). Maximum likelihood bootstrap analyses were performed using 1000 bootstrap replicates to estimate support settings. Convergence between tree topologies was confirmed by examining log likelihood values across searches. DendroPy (Sukumaran and Holder, 2010) was used to describe the parameters of the best Garli-generated tree and to construct a majority rule consensus tree from bootstrapped Garli repetitions.

The software BEAST version 2.2.1 (Bouckaert *et al.,* 2014) was used to estimate a species tree using the information from both genes. Two independent runs were performed using MCMC simulations for 3×10^7 generations, with parameters sampled every 1000th generation. Burn-in was set to 3000 (corresponding to 10% of the total samples in each run). Results obtained with BEAST were checked in TRACER v1.5 (Rambaut and Drummond, 2013) to determine adequate burn-in. I specified a relaxed clock with an uncorrelated lognormal distribution (Drummond *et al.,* 2006). Consensus trees were visualized in Figtree version 1.4.2 (Rambaut, 2012) and posterior modifications, such as insertion of posterior values and colouring of branches, were performed with Inkscape version 0.48 (www.inkscape.org).

2.6 Molecular clock and estimation of divergence times

The program BEAST was also used to infer the time to the most recent common ancestor (TMRCA) of specific lineages within *A. lunulatus* (see section “Results”). I used only the COI dataset with the same partition scheme (the input file was appropriately formatted with the BEAUti utility included in the same program package) under a relaxed clock model, because there are no estimates of substitution rates for the 28S gene for Decapods.

To date the divergence between the major *Acanthonyx* clades, we used two substitution rates (0.66% and 2.33%) estimated by Schubart *et al*. (1998) for Jamaican crabs. The estimated times of divergence should be taken with caution since there are

no calibration points for *Acanthonyx* in the NE Atlantic. The main purpose of these estimation is to have an idea of whether or not divergence events in *Acanthonyx* pre- or post-date the MSC. Two independent runs of $3x10^7$ generations were computed, with parameters sampled every 1000th generation. Burn-in was set to 3000 (corresponding to 10% of the total samples in each run).

3. Results

3.1 Morphologic identification

Based on the observation of the previous selected morphological characters (see Table 3) it was possible to distinguish at least two morphologically distinct lineages within *A. lunulatus sensu* Ng *et al.* (2008), hereafter referred to as *A. lunulatus sensu lato*. One includes specimens that fit the original description of Risso (1816), hereafter called *A. lunulatus sensu stricto* (or simply *A. lunulatus*) and range from the Mediterranean to the Atlantic coasts of Northern Africa, including the Canaries and the Madeira archipelagos, but excluding the Azores and Cape Verde. The other, includes specimens from the Azores and Cape Verde, and correspond to *A. brevifrons* Milne-Edwards 1869, *sensu* Manning and Holthuis (1981), hereafter referred to as *A. brevifrons*. The prominent morphologic differences found between these two species are presented in Table 5

A. brevifrons is distinguished from *A. lunulatus* mainly by the number of lateral lobes, bearing two instead of three in each side of the carapace. It also differs by having shorter rostral teeth, for being smother and for having a V-shaped rostral sinus, instead of a U-shaped one (see Figure 5 and Figure 6). Other morphological characters analysed proved to be highly variable and did no permit a clear differentiation between these two forms.

Table 5 - Morphological differences found between *Acanthonyx lunulatus* and *Acanthonyx brevifrons.*

	Acanthonyx lunulatus	***Acanthonyx brevifrons***
Lateral lobes	Three lateral lobes	Two lateral lobes
Rostral sinus	U- shaped	V-shaped
Rostral teeth	Longer	Shorter
Carapace	With tufts of setae	Usually smoother

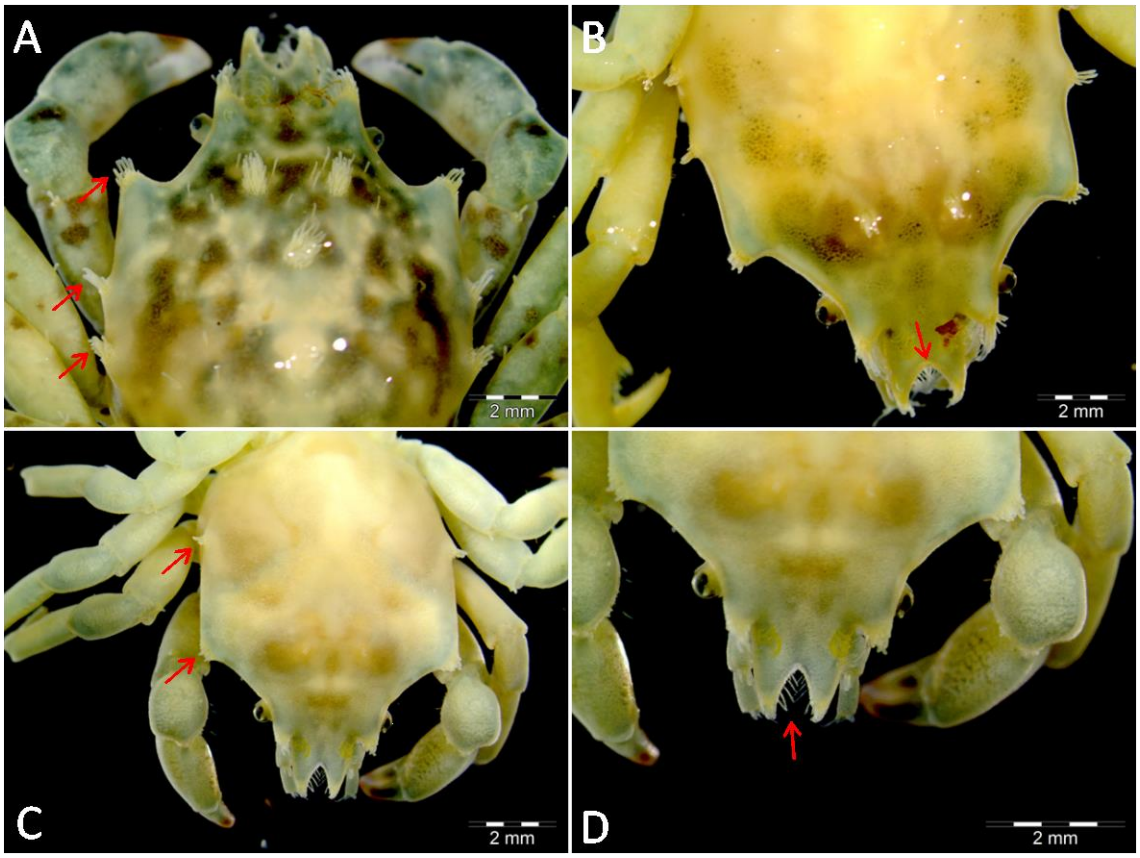

Figure 5 - Dorsal view of *Acanthonyx lunulatus* (A/B) and *Acanthonyx brevifrons* (C/D), showing the two main morphological differences between them. The specimens of *A. lunulatus* have three lateral lobes on each side of the carapace (A) and *A. brevifrons* shows only two lateral lobes (C). *A. lunulatus* exhibits a U-shaped rostral sinus (B), while *A. brevifrons* has a V-shaped rostral sinus (D).

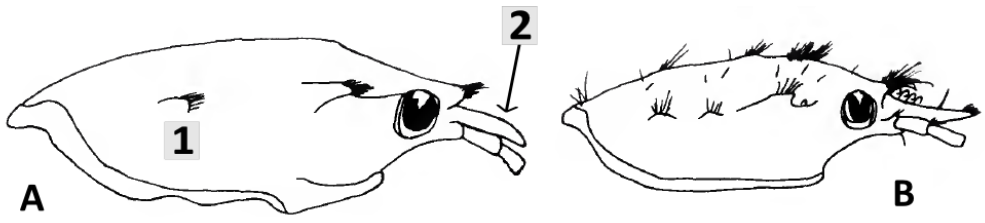

Figure 6 - Carapaces of A) *Acanthonyx brevifrons* and B) *Acanthonyx lunulatus* in lateral view. 1 – Lateral lobe; 2 – rostral teeth (Adapted from Manning and Holthuis (1981)).

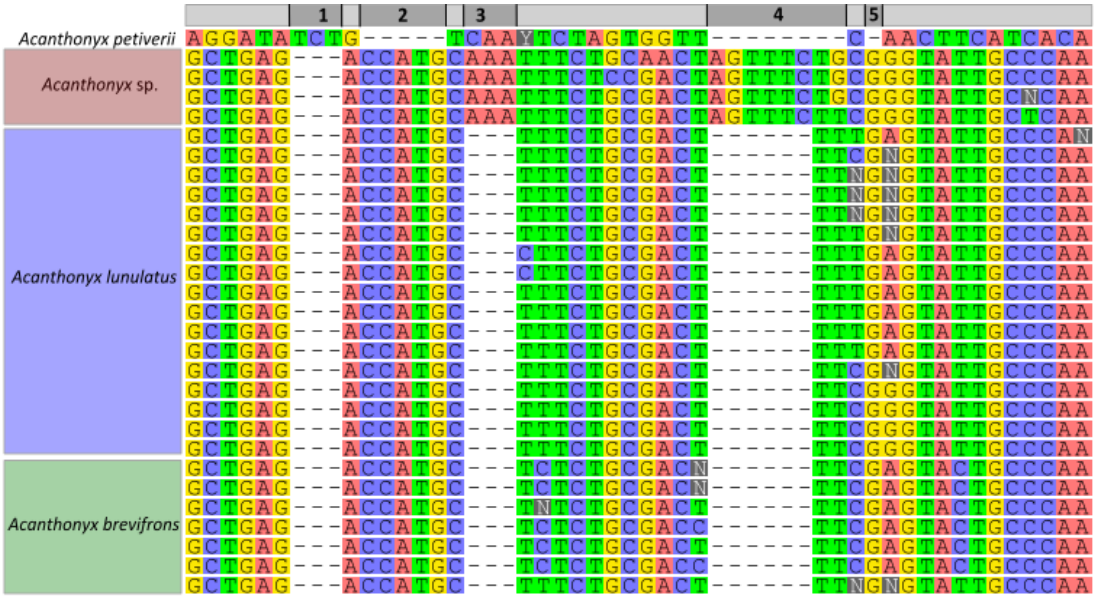

Figure 7 - Indels observed in the 28S alignment.

3.2 Alignment and sequence data

The two genes, COI and 28S, were amplified for 197 and 28 *Acanthonyx* individuals, respectively. The alignment of COI did not raise any difficulties. However, for the nuclear gene, some indels were found (Figure 7): one insertion was exclusive of an yet unidentified *A. lunulatus* lineage, and another was shared between this lineage and the outgroup *A. petiverii.* Two further deletions and an insertion were common to all *A. lunulatus* lineages by contrast with *A. petiverii*. The program 2matrix (Salinas and Little, 2014) was used to codify them into a binary partition.

The alignment of the COI and the 28S datasets had a total length of 614 and 503 bp, and included 100 and 10 unique haplotypes, respectively. The alignment of the concatenated dataset had a total of 1131 bp and included 28 original, plus four outgroup species. The 28S and the concatenated datasets were extended with an indel partition (5 characters). No stop codons were found when the amino acid translation was examined, so there was no reason to believe that these results came from a nuclear pseudogene.

3.3 Haplotype Network Reconstruction

Phylogenetic relationships between haplotypes are depicted in Figure 8. The construction of the haplotype network for all sequenced COI data retrieved three separate networks that could not be connected using the 95% parsimony connection limit. The number of haplotypes discovered ranged from 16 in *A. brevifrons* to 66 in *A. lunulatus*. The “new” lineage, hereafter referred to as *Acanthonyx* sp. displayed 18 unique haplotypes.

A. brevifrons presented some relative distantly related haplotypes (Figure 8-A), that are all exclusive from Cape Verde and Azores and do not share any haplotypes with the other two networks. The *Acanthonyx* sp. network had a simple star-like shape, with one very common haplotype surrounded by several low-frequency haplotypes, with a maximum distance of three mutation steps (Figure 8-B). In this case, just a few haplotypes belonged to Mediterranean locations (Zaouia), as most came from the Atlantic coasts of Morocco (Temara; Asilah; Le Falouque) and Spain (Conil). The *A. lunulatus* network (Figure 8-C) is highly branched with intricate patterns. It has a big common haplogroup that contains haplotypes from most of the locations sampled, and a second less common haplogroup, nine mutations apart, which as a similar geographical extent. The remain haplogroups comprise mostly low frequency and private haplotypes, many separated by a single mutation steps from the former two, but also forming a distant branch, which includes only individuals from the Mediterranean with the exception of an haplotype from Selvagens. Interestingly, if this individual is not considered, the Selavgens/Canaries haplotypes form a slightly distinct branch separated by at least six mutation from the principal haplogroup, and they share some similarities with haplotypes from Morocco Alboran and not with those from Atlantic Morocco.

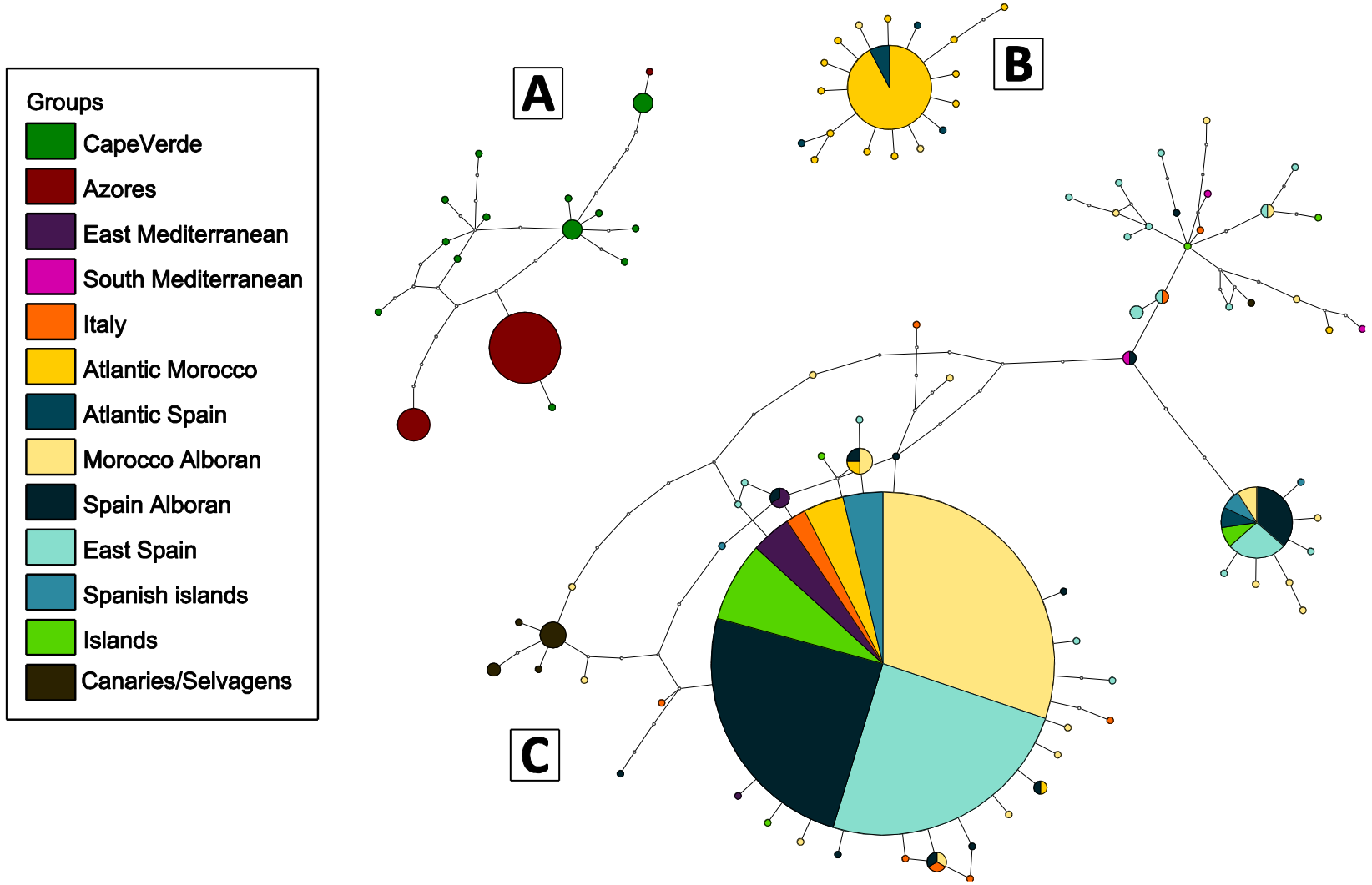

Figure 8 - Haplotype networks (95% parsimony connection limit) for all sequenced data. Haplotype networks (A), (B), and (C) correspond to haplotype relationships between individuals of *Acanthonyx.* A) *Acanthonyx brevifrons;* B) *Acanthonyx sp.;* C) *Acanthonyx lunulatus.*

Table 6 - Estimates of genetic diversity and neutrality tests for the mtDNA COI gene for each sampling site. N – Number of individuals; H – observed number of haplotypes; Hd- haplotype diversity; π – nucleotide diversity. In blue are indicated the significant values (P<0.05).

Species	Localities	Country	N	H	Hd	π	Tajima's D	Fu's FS
Acanthonyx brevifrons	Mindelo	Cape Verde	17	13	0.9559	0.008143	-1.41224	-5.13144
	Monte da Guia (Azores)	Portugal	4	2	0.5000	0.004886	-0.80861	2.94444
	Santa Maria (Azores)	Portugal	13	3	0.5641	0.006515	-0.18601	4.90298
	Azores (pooled)	Portugal	17	3	0.5221	0.005856	-0.24613	5.27666
Acanthonyx sp.	Asilah	Morocco	13	5	0.5385	0.001253	-1.86311	-2.44337
	Temara	Morocco	5	4	0.9000	0.003257	-1.12397	-1.01160
	La Falouque	Morocco	6	6	1	0.002714	-1.33698	-4.89066
	Zaouia	Morocco	2	2	-	-	-	-
	Conil	Spain	4	4	1	0.003257	-0.78012	-1.87180
Acanthonyx lunulatus	Algiers	Algeria	1	1	-	-	-	-
	L'Île-Rousse	France	2	2	-	-	-	-
	Molivos	Greece	1	1	-	-	-	-
	Temara	Morocco	2	2	-	-	-	-
	Azla	Morocco	3	3	-	-	-	-
	Cala Iris	Morocco	4	4	1	0.013844	1.14722	0.14168
	Dos Torres	Morocco	1	1	-	-	-	-
	Asilah	Morocco	3	3	-	-	-	-
	Al Hoceima	Morocco	7	2	0.2857	0.001861	-1.43414	2.04698
	Raselma Nador	Morocco	9	7	0.9167	0.010767	-0.26628	-0.59696
	Zaouia	Morocco	12	10	0.9545	0.011228	-0.88980	-2.52685
	Cala Blanca	Spain	3	3	-	-	-	-
	Cap d'Artrutx	Spain	3	2	-	-	-	-
	Cabo de Gata	Spain	5	4	0.9000	0.008469	-1.21039	0.55218
	Cala del Tío Ximo	Spain	22	14	0.8745	0.011767	0.04942	-2.45712
	Cala de sant Vicenç	Spain	1	1	-	-	-	-
	Chipiona	Spain	1	1	-	-	-	-
	Alboran Island	Spain	4	3	0.8333	0.001629	-0.70990	-0.88730
	Benalmadena	Spain	6	3	0.6000	0.005429	-1.43477	2.03553
	Denia	Spain	1	1	-	-	-	-
	El Hierro	Spain	1	1	-	-	-	-
	Herradura	Spain	9	7	0.9444	0.012215	0.09440	-0.32362
	Cabo de Palos	Spain	8	7	0.9643	0.009772	-0.14223	-1.49337
	Peniscola	Spain	3	3	-	-	-	-
	Torreguadiaro	Spain	4	3	0.8333	0.010315	-0.32685	1.87762
	Selvagens	Portugal	8	5	0.7857	0.007213	-1.67410	0.53243
	Porto Torres	Italy	1	1	-	-	-	-
	Giovinazzo	Italy	4	4	1	0.012486	-0.64018	0.01708
	Santa Marinella	Italy	1	1	-	-	-	-
	Formiggi beach	Italy	1	1	-	-	-	-
	Monopoli	Italy	1	1	-	-	-	-
	Simius	Italy	5	4	0.9000	0.013355	0.03603	1.28096
	Tropea	Italy	1	1	-	-	-	-
	Bizerte	Tunisia	2	2	-	-	-	-
	Çirali Limani	Turkey	3	3	-	-	-	-
	Fethiye	Turkey	1	1	-	-	-	-

3.4 Diversity indices, tests of selective neutrality

Estimates of genetic diversity measures and neutrality tests are provided in Table 6 for *A. lunulatus, Acanthonyx* sp. and *A. brevifrons*. The highest values of nucleotide diversity were found in *A. lunulatus* (e.g. 0.013844 and 0.013355). For the Atlantic clades - *Acanthonyx* sp. and *A. brevifrons* - lower values of nucleotide diversity were obtained, although they were slightly higher for the latter clade. All species showed generally high haplotype diversity values with the exception of *A. lunulatus* at the location of Al Hoceima (0.2857). Neutrality tests for *A. lunulatus* and *A. brevifrons* were mostly non-significant with the exception of two Tajima's D test values for Benalmadena and Selvagens and one Fu's FS test value for Mindelo, respectively. On the other hand, Fu's FS tests for the *Acanthonyx* sp. clade yielded significant values for all locations with the exception of Temara, suggesting a recent expansion or a selective sweep in this clade.

3.5 Phylogenetic relationships within NE Atlantic *Acanthonyx*

The K2P- and uncorrected p-distances among selected individuals ranged from 0 to 0.18 and 0 to 0.16, respectively (Table 7). Overall, they were similar for the same pairwise comparison, with K2P distances being usually larger by 1%. Distances between the three identified lineages of *A. lunulatus sensu lato* were 0.03 between *A. lunulatus* and *Acanthonyx* sp. and 0.07 between *A. brevifrons* and the other two. A sequence from the literature (Windsor and Felder, 2014) attributed to *A. lunulatus* (KF452903.1) suggests a misidentification, as it is similar (K2P=0.01) to *A. brevifrons* group. Distances between the three clades and the outgroups (*A. dissimulatus*, *A. petiverii* and *A. scutiformis*) ranged from 0.13 to 0.18. Notably, genetic distances show only two distinct groups within the outgroups, separated by a distance of 0.07 (values in blue and green in Table 7). So, because outgroups represent three named species, these results indicate that at least one of them is wrongly identified.

Table 7 - COI K2p-distances (the lower-left values) and uncorrected p-distances (the upper-right values) between *Acanthonyx brevifrons* (green background), *Acanthonyx* sp. (pink background), *Acanthonyx lunulatus* (blue background) and outgroups. Values in blue and green show the differences within and between *A. scutiformis, A. dissimulatus* and *A. petiverii*, respectively.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. 2848_PT_MGU		0.00	0.01	0.07	0.06	0.06	0.07	0.07	0.07	0.06	0.01	0.13	0.12	0.12	0.12	0.12	0.13	0.13	0.13
2. 3304_PT-STM	0.00		0.01	0.07	0.06	0.06	0.07	0.07	0.07	0.06	0.01	0.13	0.12	0.12	0.12	0.12	0.13	0.13	0.13
3. 2906_CV_MIN	0.01	0.01		0.06	0.06	0.06	0.07	0.07	0.07	0.06	0.01	0.13	0.12	0.13	0.12	0.12	0.13	0.13	0.13
4. 2292_ES-CON	0.07	0.07	0.07		0.00	0.06	0.03	0.03	0.03	0.02	0.06	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
5. 2291_ES-CON	0.07	0.07	0.07	0.00		0.00	0.03	0.03	0.03	0.02	0.06	0.15	0.15	0.14	0.14	0.15	0.15	0.15	0.15
6. 1807_MA-ASS	0.07	0.07	0.07	0.00	0.00		0.03	0.03	0.02	0.02	0.06	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
7. 1567_IT-CSV	0.07	0.07	0.07	0.03	0.03	0.03		0.02	0.00	0.01	0.07	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
8. 1835_ES-CTX	0.08	0.08	0.07	0.03	0.03	0.03	0.02		0.02	0.01	0.07	0.16	0.16	0.15	0.15	0.16	0.15	0.15	0.15
9. 1860_MA-ZAO	0.07	0.07	0.07	0.03	0.03	0.03	0.00	0.02		0.01	0.06	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
10. *Acanthonyx lunulatus* JQ305885.1	0.07	0.07	0.06	0.03	0.02	0.02	0.02	0.01	0.01		0.06	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
11. *Acanthonyx lunulatus* KF452903.1	0.01	0.01	0.01	0.07	0.06	0.06	0.07	0.08	0.07	0.07		0.13	0.12	0.12	0.12	0.12	0.13	0.13	0.13
12. *Acanthonyx scutiformis* KC695767.1	0.14	0.14	0.14	0.17	0.16	0.17	0.17	0.18	0.17	0.17	0.14		0.00	0.00	0.00	0.00	0.07	0.07	0.07
13. *Acanthonyx petiverii* KC695771.1	0.14	0.14	0.14	0.16	0.16	0.16	0.17	0.18	0.17	0.17	0.14	0.00		0.00	0.00	0.00	0.07	0.07	0.07
14. *Acanthonyx petiverii* KC695773.1	0.13	0.13	0.14	0.17	0.16	0.16	0.17	0.17	0.17	0.16	0.13	0.00	0.00		0.00	0.00	0.07	0.07	0.07
15. *Acanthonyx petiverii* KC695775.1	0.13	0.13	0.13	0.16	0.16	0.16	0.17	0.17	0.17	0.16	0.13	0.00	0.00	0.00		0.00	0.07	0.07	0.07
16. *Acanthonyx petiverii* KC695772.1	0.14	0.14	0.14	0.16	0.16	0.16	0.17	0.18	0.17	0.17	0.14	0.00	0.00	0.00	0.00		0.07	0.07	0.07
17. *Acanthonyx petiverii* EU682854.1	0.15	0.15	0.15	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.15	0.07	0.07	0.07	0.07	0.07		0.00	0.00
18. *Acanthonyx petiverii* EU682855.1	0.15	0.15	0.15	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.15	0.07	0.07	0.07	0.07	0.07	0.00		0.00
19. *Acanthonyx dissimulatus* KC695765.1	0.15	0.15	0.15	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.15	0.07	0.07	0.07	0.07	0.07	0.00	0.00	

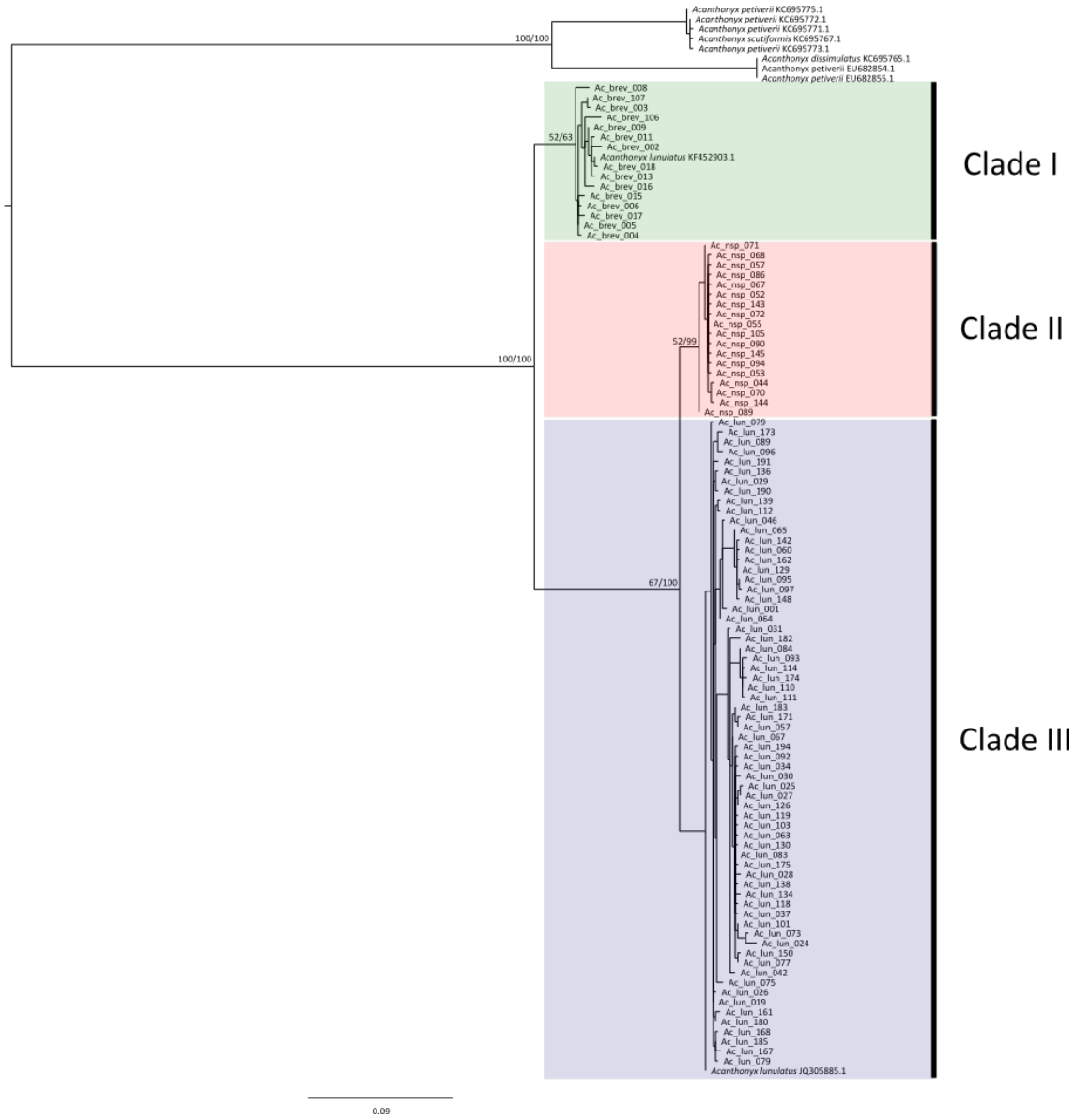

Figure 9 - Maximum-Likelihood tree obtained for mitochondrial gene (COI). Values at the nodes correspond to bootstrap support and Bayesian posterior probability, respectively.

For the phylogenetic analysis, I used the best common models of nucleotide substitution implemented on MrBayes, Garli and BEAST to compare resulting trees. Results from JModelTest, with the best and the used models, are depicted in Table 8.

Table 8 - JmodelTest results and models (ranks in parenthesis) used in the analysis

Dataset	Single		Concatenated	
	COI	28S	COI	28S
Best model	TIM2+I+G	TrN	TrN+I	TrN
Used	GTR+G (7th)	HKY (2nd)	GTR+I (4th)	HKY (2nd)

Phylogenetic analysis for all datasets rendered trees with similar overall topologies, differing only in the position of a few haplotypes within inner groups and also in the statistical support of the clades (Figures 9-11). Bootstrap support (BP) from ML and Bayesian posterior probability (PP) values were congruent with each other. The latter were usually higher than the former but it is known that PP are typically higher than BP values (Suzuki *et al*., 2002).

Acanthonyx lunulatus sensu lato was found to be monophyletic in all analysis (Figure 10). Also, all phylogenetic analysis recovered the previous results from the haplotype network reconstruction, revealing three major clades all of which had moderate to relatively high bootstrap support: *A. brevifrons sensu stricto* (Clade I), *Acanthonyx* sp. (Clade II), and *A. lunulatus* (Clade III). The *A. brevifrons* clade comprised just Cape Verde and Azores specimens. This clade appeared as the most basal for mitochondrial (COI) and concatenated (COI+28S) datatsets (Figures 9 and 11), but is weakly supporter in the former. Clade II, the *Acanthonyx* sp. clade, included Atlantic forms from Morocco and Spain and a few haplotypes from the Mediterranean Morocco (near the Strait of Gibraltar). This Clade was highly supported in all analysis and for all datasets, except in the ML analysis based on the mitochondrial gene (52% bootstrap value; Figure 9). Finally, the *A. lunulatus sensu stricto* included all haplotypes found on all locations except from the Azores and Cape Verde, and emerged as monophyletic in

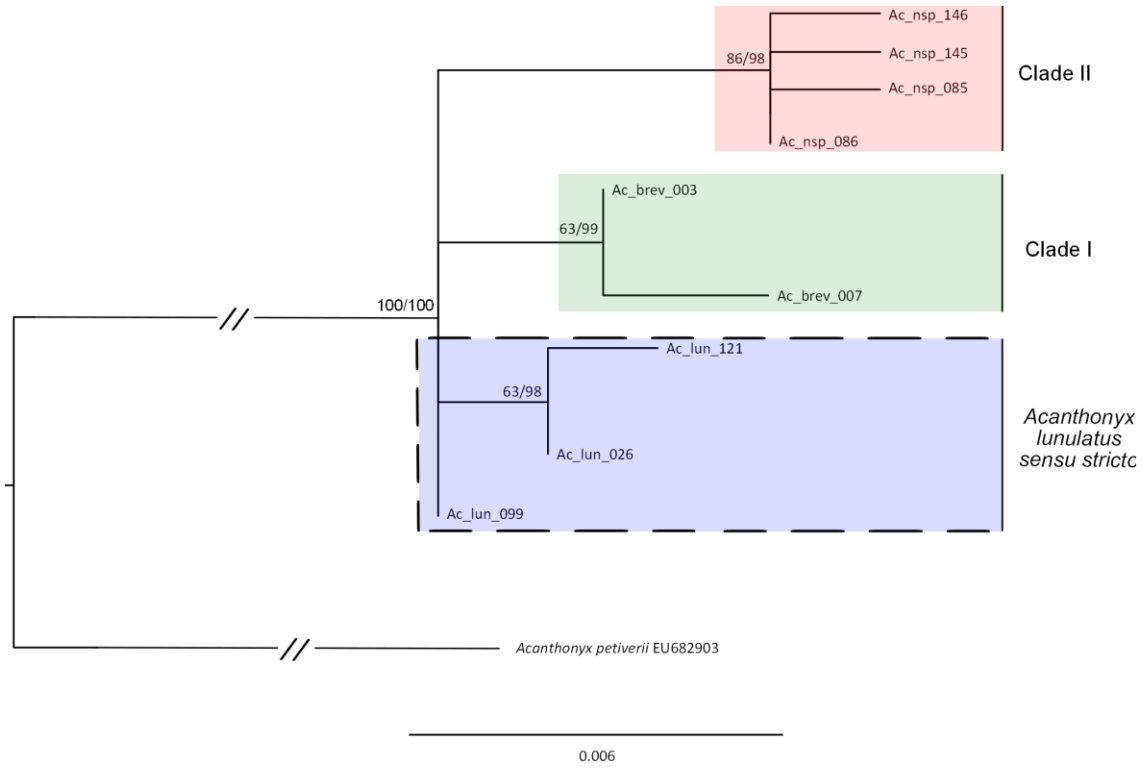

Figure 10 - Maximum-Likelihood tree obtained for the 28S rRNA gene. Values at the nodes correspond to bootstrap support and Bayesian posterior probability, respectively.

all analyses except for the 28S dataset. When using COI and concatenated dataset (Figures 9 and 11), *Acanthonyx* sp. and *A. lunulatus*, appeared as sister species with moderate or high support, respectively. However, this monophyly must be interpreted with caution, because of the low ML support found for inner clades when using the combined nuclear and mitochondrial datasets (see value of 22% into Clade III, Figure 11).

3.6 Estimation of divergence times

Estimates for the time to the most recent common ancestor (TMRCA) of each clade, as well as their 95% credibility intervals, were obtained for two different substitution rates (0.66% and 2.33%) separately. By using the lowest and highest values from the two confidence intervals obtained for each divergence event, the origin of the Atlantic-Mediterranean clades of *Acanthonyx* between *A. brevifrons* is estimated to be within 0.3653 – 1.7195 Mya. The TMRCA between *Acanthonyx* sp. and *A. lunulatus* is

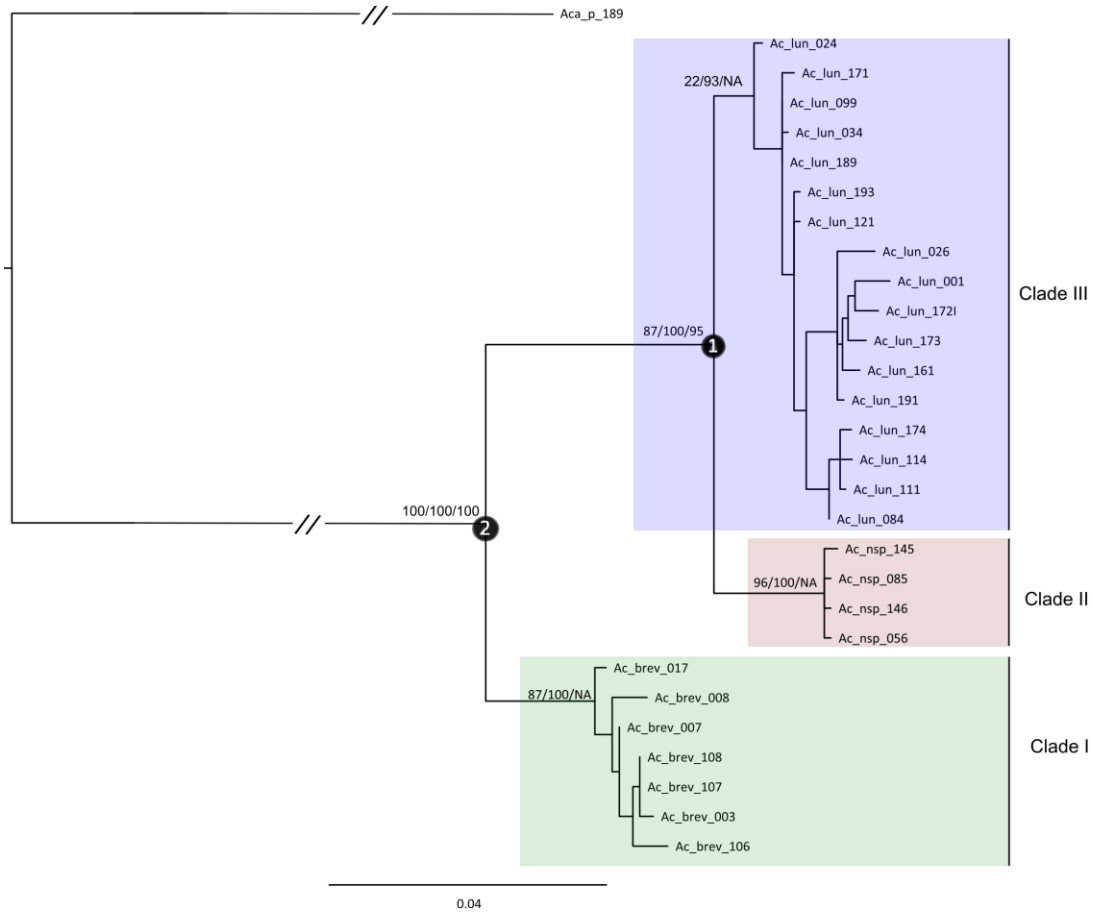

Figure 11 - Maximum-Likelihood tree obtained for the concatenated dataset (COI + 28S). Values at the nodes correspond to bootstrap support (ML) and Bayesian posterior probability for BI and the species tree, respectively.

estimated between 0.6031 and 4.1314 Mya. So, the divergence between *A. lunulatus* – and *Acanthonyx* sp. occurred at most about 1.7195 Mya (node 1 in Figure 11) and that between *A. brevifrons* – *A. lunulatus* + *Acanthonyx* sp. at most about 4.1341 Mya (node 2 in Figure 11). Therefore, data suggests that both lineages diverged after the MSC.

4. Discussion

The present results show that the prevailing taxonomic concept of *Acanthonyx lunulatus sensu* Ng *et al*. (2008) should be revised. This concept includes a single species ranging from the Mediterranean to southern Africa, including all the Macaronesian archipelagos. However, the combination of current genetic data with known morphological traits suggest that *A. lunulatus* should be viewed at least as a complex of species. Preliminary TCS analysis of the mtDNA data produced three unlinked networks at a connection limit of 95% which, according to Hart and Sunday (2007), is a good indicator for the presence of cryptic species. With the exception of the 28S dataset, phylogenetic analysis always recovered three monophyletic clades, although with different statistical support. Average K2P- and uncorrected p-distances between the three linages of *Acanthonyx* provide further evidence for the current interpretation of their taxonomical status. According to Silva *et al*. (2011), levels of intragenus divergence of the COI gene in Decapoda range from 5% to 32%. My results show that the divergence between *A. lunulatus* and *A. brevifrons* is 7%, which is still within the mentioned interval, whereas between *A. lunulatus* and *Acanthonyx* sp. is only 3%. These marginal values are not totally unexpected given the likely recent diversification of the group. Even though individuals belonging to each lineage were fairly similar genetically, there are sets of specimens from each lineage that clearly display higher inter-lineage similarities than others. This signal was observed either between *A. lunulatus* and *A. brevifrons* for the COI dataset, and between the former and *Acanthonyx* sp. for the 28 dataset. Interestingly, these sets reveal no congruent geographical pattern, which may result from the high dispersion ability of *Acanthonyx*. The lower differentiation of some individuals belonging to distinct lineages may explain the small branch support in both ML and BI analyses, and the lack of monophyly of *A. lunulatus* in the 28S dataset.

The taxonomic view that best fits the current genetic evidence is the one proposed by Manning and Holthuis (1981). Hence, all specimens from Mediterranean Sea and most of those found in the NE Atlantic localities, including the Macaronesian archipelagos of Canaries and Madeira, should be classified *as A. lunutatus*. The specimens from Cape Verde and the Azores correspond to the description of *A. brevifrons*, which should be recognized as a good species. The third lineage (*Acanthonyx* sp.) does not fit any described species or sub-species known so far, and should be viewed as an unrecognized taxonomic entity or a putative cryptic species of *Acanthonyx*.

4.1 Distribution of *Acanthonyx brevifrons*

The taxonomic status of *A. brevifrons* Milne-Edwards, 1869 has never been consensual. Some authors considered it as a variety of *A. lunulatus* (e.g. Chapman and Santler, 1955, Emparanza *et al*., 2007; Ng *et al*., 2008) while others viewed it as distinct species (Manning and Holthuis, 1981). A possible explanation for these contrasting views stems from the superficial morphological similarities between these species, coupled with the usage of highly variable diagnostic characters to distinguish them. Due to the early synonymization of both species, occurrence records from the literature make it difficult to establish their actual distribution.

Milne-Edwards (1869) described *A. brevifrons* from the bay of St. Vicente (Cape Verde). Later, Milne Edwards and Bouvier (1900) reported this species from the Azores (Santa Maria Island). However, in this work they also reported the presence of *A. lunulatus* in Cape Verde, citing a record from the "Challenger" expedition without any further reference. This record can only be attributed to Miers (1886) who, based on a single immature female, noted that "It approaches the species or variety *Acanthonyx brevifrons*, A. Milne Edwards, in the form of the front, but there are indications of three antero-lateral teeth, and the carapace, as in *Acanthonyx lunulatus*, bears several tufts of setae". Hence, Miers did not explicitly synonymized both species, an opinion later followed by many authors (e.g. Balss, 1922; Monod, 1933), including Bouvier (1940) himself, in his seminal work "Faune de France".

D'Acoz (2001) examined Azorean specimens of what he called *A. lunulatus* and discussed the possibility of the presence of *A. brevifrons* in Azores because the specimens had only two well-defined lateral lobes with a small prominence hinting at the presence of a third one. Although he admitted that the specimens examined were fairly variable morphologically but formed a cohesive group, he opted to classify the Azorean specimens as *A. lunulatus* given the lack of acceptance of *A. brevifrons*. In an early work also carried in the Azorean archipelago, Paula *et al*. (1992) identified both *A. brevifrons* and *A. lunulatus*, but remarked that the latter specimens had intermediate features between the "true" Mediterranean *A. lunulatus* and *A. brevifrons.* All *Acanthonyx* from the Azores and Cape Verde analyzed in the present work belong to a distinct genetic clade but include both the typical morphotype of *A. brevifrons* (two lateral lobes) as well as the "intermediate" morphotype with two lateral lobes and a hint of a third median one.

Manning and Holthuis (1981) also discussed the presence of *A. lunulatus* in Cape Verde, although they did not found any direct evidence of this species in the material they collected. They did not dismiss such occurrence because their specimens were

collected at 75-180m depths, whereas previous records attributed to *A. lunulatus* were all from specimens collected at shallow waters and such non-overlapping habitats could explain their occurrence in sympatry. Specimens collected for this work at Cape Verde and the Azores all come from shallow waters (0-2m deep) and none is genetically similar to *A. lunulatus* linage. Hence, since there is no factual evidence of any *Acanthonyx* found in the Azores and Cape Verde with a true median lobe bearing a tuff of setae, the presence of *A. lunulatus* in both archipelagos is dismissed for the time being.

The mtDNA haplotype network that clusters the Azorean and Cape Verde specimens shows a clear separation of these archipelagos, as there are no shared haplotypes between them. However, the Azorean haplotypes only differ from the Cape Verde ones by a few mutations. Whereas at Cape Verde haplotype diversity is quite high (Hd = 0.96), meaning that no two individuals share the same haplotype, in the Azorean islands only three different haplotypes were found (Hd = 0.522) for the same sample size (n = 17). Furthermore, the Azorean haplotypes do not form a distinct branch out the Cape Verde network but are related to highly differentiated haplotypes from the latter archipelago. Hence, data strongly suggests that haplotype diversity in the Azores is not created in loco, hinting to a recent colonization of this archipelago from Cape Verde. Whether this colonization is historical or contemporary (eventually human-mediated) cannot be answered by the current data given the high haplotype and nucleotide diversity of COI in *A. brevifrons,* and lack of spatial coverage.

4.2 A cryptic lineage of *Acanthonyx* in NE Africa

Several individuals from the Atlantic coasts of southeast Spain and northeast Morocco, and some Moroccan Mediterranean localities, formed a distinct, monophyletic and well-supported clade. Their haplotype network displayed a characteristic star-like structure, which suggests recent demographic expansion. This conclusion is supported by the Fu's Fs neutrality tests, which were significant for all populations but Temara. A detailed morphological inspection of all individuals of this clade did not reveal any characteristic that allowed their distinction from *A. lunulatus*. Comparison with the description of a southern species known from the islands of Ascension and Saint Helena – A. *sanctahelenae* Chace, 1956 – was also inconclusive.

K2P- and uncorrected p-distances between *A. lunulatus* and *Acanthonyx* sp. are within intra-species diversity in Majoidea (Silva *et al*., 2011), but a 3% divergence level has already been accepted as a threshold for cryptic species in other crustaceans (Radulovici *et al*., 2009). Notwithstanding, the strongest genetic difference between

Acanthonyx sp. and the other two lineages was detected in the nuclear gene through the presence of indels. *Acanthonyx* sp. shares a deletion with *A. lunulatus* and *A. brevifrons* when compared to the outgroup *A. petiverii*, but differs from the former two by having two insertions, one if which is also observed in *A. petiverii*. Usage of indels in phylogenetic analysis should be taken with caution but it is important not to ignore this kind of information because it can improve the interpretation of the results (Zhang and Hewitt, 2003). Nuclear ribosomal genes are known to be highly uniform within species, but may vary significantly between species (Eickbush and Eickbush, 2007). I found no evidence of heterozygosity in the 28S of both *A. lunulatus* and *Acanthonyx* sp., which would be revealed as a well formed chromatogram up to the first indel and unreadable from there onwards, due to the overlapping of a shifted sequence. The absence of shared haplotypes at cytoplasmic and nuclear genes is good evidence of reproductive isolation, which is surprising given that the two lineages occur in sympatry, and cryptic species tend not to co-occur (Vodă *et al.,* 2015). A greater sampling effort at the entrance of the Mediterranean and the usage of more molecular markers will help to determine the taxonomic status of this distinct *Acanthonyx* group.

4.3 The taxonomy of the genus *Acanthonyx*

The genus *Acanthonyx* is known to occur world-wide, but the present work, although focused only on the NE Atlantic region using a presumably single species, unveils potential problems with the taxonomy of this morphologically variable group of spider-crabs. The reason why I changed the initial goal of my work – the phylogeography of *A. lunulatus* in the Northeast Atlantic and the Mediterranean – stems from a sequence published in GenBank (KF452903) and attributed to that species, but which differed by 8% from my own data. This sequence was used by Windsor and Felder (2014) as an outgroup for the phylogenetic analysis of the family Mithracidae (Epialtidae) and corresponds to a specimen sampled at Cape Verde. In light of the current molecular data, this specimen belongs to *A. brevifrons*. Whether the authors adopted explicitly the taxonomic concept of Ng *et al.* (2008) or were unaware of the work of Manning and Holthuis (1981) remains to be known.

Usage of K2P- and uncorrected p-distances of COI to estimate average levels of divergence between good species of *Acanthonyx* also revealed some problematic issues with sequences deposited on GenBank. For example, while studying Brazilian epialtids, Gomes (2013) found no divergence between a sequence that she attributed to *A. scutiformis* (KC695767.1) and those of *A. petiverii*, and proposed the synonymization

of the former, although the name still remains at GenBank. Moreover, she found that *A. dissimulatus* (KC695765.1) differed from most of her *A. petiverii* samples by 7%, but it was identical to one GenBank sequence of *A. petiverii* sampled in the USA (Gulf of Mexico) and used by Hultgren and Stachowicz (2008) on the phylogeny of the Majoidea, plus two sequences of *A. petiverii* from Mexico. She suggested the synonymization of *A. dissimulatus* with *A. petiverii*, although data clearly hints at a possible misidentification of the northern and central American specimens. In their redescription of *A. petiverii*, Emparanza *et al.* (2007) compared the specimens collected in Chile with the descriptions of Chace (1966) for Martinique and the Virgin Islands. They noted some morphological differences between both groups, namely on the disposition of tubercles on the carapace, but they refrained from discussing the conspecific status of Pacific and eastern Atlantic *A. petiverii* populations without any further re-examination or new evidence.

Most of the problems mentioned result from the difficulty in establishing good diagnosis for the highly variable species of *Acanthonyx,* and a comprehensive revision of this genus is still needed. Although many details have been unraveled for the *A. lunulatus* group, some doubts still remain regarding its distribution. Given the levels of genetic differentiation observed, its distributional range – from the Mediterranean southwards to Namibia – should be investigated. There are few known records of *A. lunulatus* for the Gulf of Guinea, but most were recently placed into two different species: *A. depressifrons* Manning and Holthuis, 1981 and *A. minor* Manning and Holthuis, 1981. Hence, the presence of *A. lunulatus* in southeast Africa is dubious, although molecular evidence for other Majoidea species with similar larval duration shows that such a wide distribution is possible (Sotelo *et al.,* 2009).

5. Conclusion and final remarks

The Decapoda is considered the most diverse group of crustaceans and large morphologic variability among species is expectable in this group. Such diversity is likely to hide a considerable number of undiscovered and highly variable species or species complexes. With the advent of molecular techniques, reports of cryptic species are steadily growing (Bickford *et al.*, 2007), and marine invertebrates are no exception (Thorpe *et al*., 2000). As of today, there are numerous examples of cryptic crustacean species that have been discovered through molecular methods (e.g. Knowlton, 1993; Machordom and Macpherson, 2004; Mathews, 2006; Tourinho *et al*., 2012) which proves the usefulness of phylogeographycal and phylogenetic analyses for taxonomic purposes. Since there are no phylogenetic studies concerning any species of *Acanthonyx* in the NE Atlantic and Mediterranean region, the present work provides important insights into the genetic diversity and differentiation within this genus.

Overall, this work shows that high genetic variation between specimens with identical morphology is common on epialtids and described species are not always distinguishable by morphological traits. Hence, it is important to combine both morphological and genetic tools to fully understand phylogenetic relations between species. One important conclusion drawn from this work is that levels of genetic and morphological variation within the NE Atlantic and Mediterranean *Acanthonyx* are high, but the same may as well apply to other congeneric species from the Pacific and eastern Atlantic oceans. As a future work, it will be important to extend molecular analyses to other species of *Acanthonyx*, increasing either sampling coverage as well as the number of marker loci (especially at the nuclear level), to clarify the phylogeny of this genus and contribute to improve its current taxonomy.

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